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Identification of parasitic diseases affecting ranched Southern Bluefin Tuna (SBT), using conventional and molecular methods

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“Nothing ever exists entirely alone; everything is in relation to everything else.” - Gautama Buddha

“The only way to make sense out of change is to plunge into it, move with it, and join the dance.” - Alan W. Watts

“I can’t change the direction of the wind, but I can adjust my sails to always reach my destination.” - Jimmy Dean

“Old friends pass away, new friends appear. It is just like the days. An old day passes, a new day arrives. The important thing is to make it meaningful: a meaningful friend - or a meaningful day.” - Dalai Lama XIV

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Statement of Co-Authorship

The following people and institutions contributed to the publication of work undertaken as part of this thesis:

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Book chapter, located in chapter 2

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Candidate was the primary author and contributed to the preparation, development and final presentation of the manuscript. Authors 1, 8 and 9 contributed in the preparation, development, detailing and final presentation of the manuscript, in particular author 8 wrote the sections about NBT in Mediterranean, author 9 the

sections about PBT in Japan and author 1 coordinated the writing and contributed some parts of introduction and SBT as well as edited the final manuscript.

Paper 1, located in chapter 4

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Candidate and author 3 were equally first authors. Candidate performed all the molecular analyses, contributing with the planning and execution of the research project and in the development and final presentation of the manuscript. Author 1 and author 3 planned the research and contributed to development of the manuscript and its final presentation. Authors 3 and 4 performed genomic analyses, author 5 contributed general laboratory assistance including DNA extraction and PCR.

Paper 2, located in chapter 5

Power C., Balli G. J., Evans, D., Nowak, B.F., Bridle, A., Bott, N.J. 2018. Detection of *Miamiensis avidus* (Ciliophora: Scuticociliata) and *Cardicola* spp. (Trematoda: Aporocotylidae) DNA in biofouling from Southern Bluefin Tuna, *Thunnus maccoyii* pontoons off Port Lincoln, South Australia. Aquaculture (submitted)

Candidate and author 6 are equally first authors. Candidate performed all the molecular analysis for the identification of *Cardicola*, contributing with the development and execution of the research project. Candidate together with all co-authors prepared, developed and refined the final manuscript. Author 6 processed samples

(including DNA extraction), analysed the samples for *Miamiensis avidus*, analysed those data and wrote these sections of the manuscript. Author 7 contributed to the planning of the experiment, set up the experiment and collected the samples. Authors 1, 2 and 3 contributed to the planning and development of the experiment.

We the undersigned agree with the above stated "proportion of work undertaken" for each of the above published (or submitted) peer-reviewed manuscripts contributing to this thesis:

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Chris Carter

Abstract

In Australia, the Southern Bluefin Tuna (SBT) industry is regarded as one of the most profitable sectors of aquaculture. Even though SBT are relatively unaffected by infectious diseases, few still have an impact in SBT production, leading to economic losses. Two of the main diseases affecting ranched SBT are infections by blood flukes (*Cardicola forsteri* and *C. orientalis)* and swimmer syndrome. Until now, lethal sampling has been used for the detection of *C. forsteri* and *C. orientalis*, and in combination with conventional diagnostic methods, they are used as the "gold standard" for the diagnosis of *Cardicola* and Scuticociliates. Therefore, in an attempt to improve sampling and diagnostic tools for health management of SBT, the focus of this thesis was to:

- Compare different lethal and non-lethal sampling methods used with conventional and molecular diagnoses for the detection of *C. forsteri* and *C. orientalis*
- Determine the presence and identify Scuticociliates in samples from SBT showing swimmer syndrome
- Determine the presence of *C. forsteri* and *C. orientalis* in biofouling samples near SBT pontoons
- Identify the potential relationship between the substrate, the age of the biofouling sample, type of biofouling organisms and depth, with the presence of *C. forsteri*, and *C. orientalis* DNA in the biofouling

To provide a better understanding of the diseases affecting ranched Bluefin Tuna, a review is presented in Chapter 2. In Chapter 3, lethal sampling and non-lethal sampling techniques as well as, conventional and molecular methods used for the diagnosis of *C. forsteri* and *C. orientalis* are compared. Lethal and non-lethal samples from SBT were collected over the course of three years. Results showed differences between lethal sampling

used along with conventional diagnosis and non-lethal sampling and molecular diagnosis. Only 37% of the heart flushes were positive and 66% of gill filaments had egg counts, identification of *Cardicola* species proved to be too challenging. For the same SBT, real-time qPCR along with species specific primers and probes were used for the detection of *C. forsteri* and *C. orientali*s. Not all PCR-based techniques allowed the detection of animals positive for adult blood flukes of eggs, such is the case for serum samples where only 3% were real-time qPCR-positive for *C. forsteri* and 1% to *C. orientalis*.

Gill mucus samples showed similar results to heart flushes (38% of the samples positive for *C. forsteri* and 28% to *C. orientalis*), nevertheless, the use of gill mucus and real-time qPCR presents the advantage of allowing the identification of *Cardicola* species. Gill snips, gill biopsies and gill filaments samples, all presented high sensitivity when compared to conventional diagnostic techniques, where 100% of gill snips and gill biopsies were positive for *C. forsteri*, 86% of the gill filaments were also positive for *C. forsteri* and 4% of gill filaments were positive for *C. orientalis.* All molecular techniques allowed differentiation between species of *Cardicola.*

In Chapter 4, 16 olfactory rosettes from wild SBT and 23 cerebrospinal fluid (CSF) samples from ranched SBT which exhibited swimmer syndrome, were collected. CSF samples positive for Scuticociliates were identified firstly using microscopy methods observing motile cells with a pyriform shape and granular appearance. All samples were tested using end point PCR combined with primers amplifying fragments of the small subunit rDNA (SSU rDNA) and the mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*), all the amplifications obtained were sequenced and compared against published data. Olfactory rosettes were considered negative for Scuticociliates as neither of them showed amplification using the SSU rDNA and *cox1* primers. Sequencing of the PCR products of the CSF samples, evidenced presence of *M. avidus*, being 100% identical to sequences of *M. avidus* previously reported. This means that those swimmer syndrome cases investigated here were associated with *M. avidus.*

In an attempt to identify possible sources of infection of *C. forsteri, C. orientalis* and *M. avidus*, and as a result of the data obtained from Chapter 3 and 4, biofouling samples plates and mesh for biofouling were placed at different depths near the tuna pontoons. Samples were placed at one and four metres of depth and collected after one and three months. Organisms were separated according to their taxonomic group and identified by molecular techniques species-specific primers and probes for real-time qPCR. The presence of *C. forsteri* and *C. orientalis* was detected in 4 samples, each one of them. *C. forsteri* was only detected in samples collected at a depth of four metres, while 75% of the positive samples collected at 1 metre depth were positive for *C. orientalis*. *M. avidus* prevalence showed an increase from 38% during the first month to 89% of the samples during the third month with no significant differences between depths.

The results of this thesis identify different non-lethal sampling techniques that could be used along with molecular analysis as a monitoring and diagnostic tool for the detection of *Cardicola* in ranched tuna. Further studies need to be performed to evaluate the use and limitation of gill mucus as a non-lethal sample. The findings from this thesis also provide evidence of the advantages PCR and real-time qPCR have over conventional diagnostic methods, allowing the identification for the first time of *M. avidus* in ranched SBT, and associating *M. avidus* to swimmer syndrome. Finally, this work contributes to the understanding of the probable reservoirs of infection of *Cardicola* and *U. nigricans* in ranched SBT.

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Explanatory note concerning thesis structure

Chapters 2, 4 and 5 of this thesis have independently been published or submitted for publication as journal articles. As a consequence, some textual and reference overlap occurs between these chapters and with the introductory material. Chapter 1 of this thesis is written as a general introduction and review of relevant topics needed to establish the experimental rationale of subsequent chapters. Chapter 6, provides a discussion integrating the discrete research chapters and general conclusions. The Aquaculture referencing style has been adopted for this thesis; however, the orthography is consistent with the commonwealth countries of Britain. The bibliography section is presented at the end of the thesis.

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Chapter one

General Introduction

1.1 Aquaculture

Over the past five decades, fish consumption has almost doubled increasing from 9.9 kg during the 1960s to approximately 20 kg per capita, reaching over 25 kg in industrialized countries (FAO, 2016a, 2016b). Currently, nearly 80% of global fish production is consumed as food, representing 17% of the animal protein intake and 6.7% of all protein supply (FAO, 2016a, World Bank, 2013). Furthermore, fish constituted near 20% of the protein intake for almost 3.1 billion people (FAO, 2016a). The Food and Agriculture Organization of the United Nations (FAO) has estimated that in 2021 at least half of the fish consumed worldwide will originate from aquaculture. It is expected that by 2030 fish supply will increase to 187 MT (OECD-FAO, 2017; World Bank, 2013).

Aquaculture is defined as the farming of aquatic organisms, including animals and plants, involving intervention with different practices during the process to increase production, such as feeding, protection, stocking, health monitoring among others. It also implies property of the stock (FAO, 1988). Aquaculture has been practiced since ancient times with the first references to fish culture dating back to 2,500 years ago in China and 800 AD for shrimp culture in Asia (Boyd and McNevin, 2014; Lucas and Southgate, 2012). In the last 75 years, aquaculture has become a more common practice, being pursued by subsistence farmers and international companies (Boyd and McNevin, 2014; FAO, 2018). Currently nearly 567 aquatic species are farmed worldwide (FAO, 2018).

Aquaculture production continues to grow, seafood being an important source of protein, vitamins, minerals and other required nutrients (FAO, 2018; Lucas and Southgate, 2012). During the 1970s, aquaculture provided approximately 3 MT of fish, in 2015 world production achieved 76.6 MT with an estimated value of USD 157 billion,

farmed aquatic animals contributed with 72 MT, representing a total production of 43.1% of total world production. At present, 80% of aquaculture production are animals low in the food chain (FAO, 2015).

Ranching is defined as the release of juvenile fish in either marine or estuarine environments, allowing the growth of the fish in their natural environment for future harvesting purposes (FAO, 2013). Ranching usually refers to migratory species but it can include other aquatic species (FAO, 2013; Isaksson, 1988). Thus ranching is when individual fish or fish schools are captured and grown in floating pontoons (Lucas and Southgate, 2012). Combined, fisheries, ranching and aquaculture practices are a source of protein and nutrition, providing at the same time income to almost 820 million people and producing combined, approximately 202 MT during 2016 (FAO, 2016c).

In ranching, predatory fish such as tuna are commonly handled on a stock-by-stock basis, meaning each stock present different characteristics and depends on natural availability, which can have an impact in the final production. For the last 15 years, ranching has been stably producing between 88.3-93.8 MT per year, reaching 92.6 MT in 2016 (FAO, 2016c).

Australian seafood production has remained constant for the last two decades, producing an approximate of 230,000 T per year and generating high value products some of which are exported in significant quantities. Most of the imported products are of low value, such as canned fish (Department of the Environment and Energy, 2016; Department of Agriculture and Water Resources, 2017). Over the last three decades the domestic seafood demand has increased exceeding the present supply. The annual estimated value of Australia's aquaculture industry and commercial fishing is approximately AUD 2.2 billion, employing around 11,600 people (Department of Agriculture and Water Resources, 2017). Even though the fishing zone is the third

largest in the world, Australian waters are depleted of nutrients, therefore production is low (Department of Agriculture and Water Resources, 2017). Production takes place mainly in regional areas of Australia, contributing to the local development. During 2015-2016, Australian fisheries have produced an estimate of AUD 439 million, representing a gross value of production (GVP) of 26% of wild-catch fisheries and 14.5% GVP of whole fisheries and aquaculture in Australia (Department of Agriculture and Water Resources, 2017).

1.2 Tuna

Globally, the biggest proportion of marine catches consists of pelagic species such as tuna (Braham and Corten, 2015). The term “tuna” comprises a broad range of different fish species, but it is commonly linked with the big, highly profitable species belonging to the genus *Thunnus* (Benetti et al., 2016). Even though their input to the global catches is low, providing approximately 40,000 t per year, they hold high value at the market (FAO, 2016a; Sumaila et al., 2015). They contribute to approximately 20% of the profits of marine fisheries, becoming one of the most regarded fish species (Arrizabalaga et al., 2015; Benetti et al., 2016; FAO, 2011), leading to an increase in tuna fishing effort and drastically reducing some tuna populations as a result (Benetti et al., 2016).

There are seven main species considered to be of high economic importance worldwide: Albacore (*Thunnus alalunga*), Northern Bluefin Tuna (*T. thynnus*), Southern Bluefin Tuna (*T. maccoyii*), Pacific Bluefin Tuna (*T. orientalis*), Yellowfin tuna (*T. albacares*), Bigeye tuna (*T. obesus*), Skipjack tuna (*Katsuwonus pelamis*) (FAO, 2011). The three species of Bluefin Tuna are Northern Bluefin Tuna (NBT), Pacific Bluefin Tuna (PBT) and Southern Bluefin Tuna (SBT) (FAO, 2011) (Table 1.1). SBT

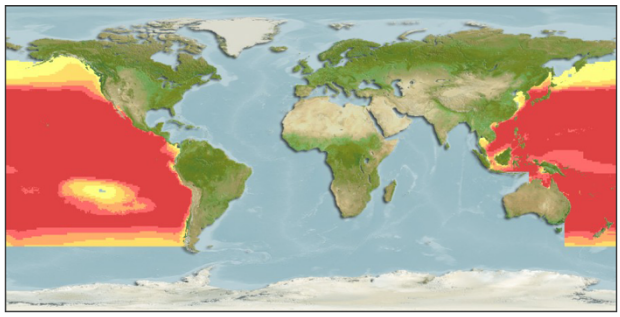
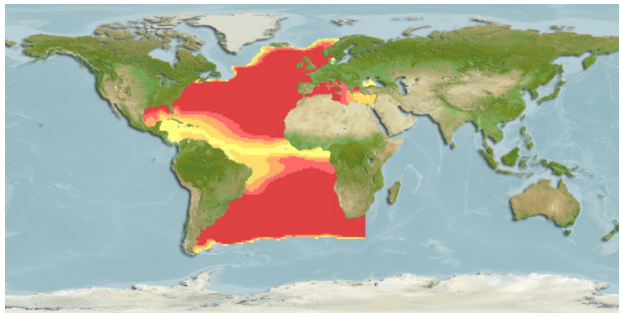
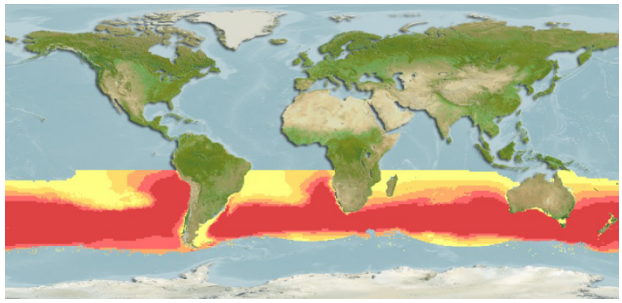
is found in the southern hemisphere (CCSBT, 2018; Evans et al., 2012; Stefano and Heijden, 2007). SBT adults can have a maximum weigh of 250 kg and measure approximately 245 cm, living up to 40 years, they spend most of their lives in waters with temperatures between 5 – 30^{o}C (CCSBT, 2018; Evans et al., 2012; Farley and Davis, 1998; Patterson et al., 2008). SBT only breed in the tropical waters south of Java, spawning in the Indian Ocean between September and April (Caton, 1991; Farley and Davis, 1998; Shiao et al., 2008) and during the months of December to April, juveniles migrate, gathering close to the ocean surface along the coast of Southern Australia and spending winter in temperatures ranging from 5 to 20^{o}C in the deep oceanic waters (CCSBT, 2018; Honda et al., 2010; Patterson et al., 2008; Shiao et al., 2008).

Pacific Bluefin Tuna are commonly found in the temperate and sub-tropical waters of the North Pacific Ocean and in the Tasman sea and in the tropical waters surrounding New Zealand (Craig et al., 2017). Spawning only happens in the West Pacific between April and August (Collette et al., 2014; Kitagawa et al., 2000). During summer, juveniles migrate to the North of the Japanese and Korean coasts, remaining there for one year (Craig et al., 2017; Kitagawa et al., 2000). Juveniles between one to three years old migrate to the North Pacific Ocean spending several years migrating along the coastal lines of Baja California in Mexico to California in the United States (Collette et al., 2014; Madigan et al., 2017) before returning to the West Pacific Ocean (Craig et al., 2017; Madigan et al., 2017). Adult PBT can reach a maximum length of 300 cm and 500 kg (Collette et al., 2014; Craig et al., 2017) the maximum age recorded has been of 26 years (Craig et al., 2017).

Northern Bluefin Tuna spend their entire lives in the temperate waters of the Atlantic Ocean, from the North Atlantic to the Mediterranean sea (Collette and Nauen,

1985; Noaa Fisheries Service, 2010; Rooker et al., 2007). They are divided into two different stocks, Eastern Atlantic and Mediterranean stock and Western Atlantic stock (Fromentin and Powers, 2005; Gordoa et al., 2009), spawning mainly at two different sites: the Mediterranean Sea and the Gulf of Mexico (Collette and Nauen, 1985; Noaa Fisheries Service, 2010). They live in depths of 500 m, occasionally diving up to 1,000 m (Collette et al., 2001). NBT can live up to 40 years, and as is the case for SBT, adults of more than eight years can weigh 200 kg and measure more than 185 cm (Collette et al., 2001; Mather et al., 1995; Tiews, 1963) increasing their size up to 400 kg and 300 cm by the age of 20 years (Noaa Fisheries Service, 2010).

Table 1.1 Distribution of Bluefin Tuna. Maps from Kaschner et al., 2016, www.aquamaps.org (accessed 3 Feb 18).

Species	Distribution	IUCN status
PBT *Thunnus orientalis*		Vulnerable
NBT *Thunnus thynnus*		Endangered
SBT *Thunnus maccoyii*		Critically endangered

Tuna ranching started at the end of the 1960s, but it was at the beginning of 1990s that ranching practices were properly established, leading to the development of the modern tuna industry (Benetti et al., 2016). In Japan, young wild tuna *Thunnus thynnus* are captured using longline fishing (Clarke et al., 2014; FAO, 2011; Ottolenghi, 2008; Stefano and Heijden, 2007), while in Australia, the main method is purse seine. Fish are then towed in a specifically designed pontoon to the ranching grounds, where they are transferred to static grow-out pontoons, and grown on a diet

based on bait fish (ASBTIA, 2015; FAO, 2011; N. T. Kirchhoff et al., 2011a). Since the 1970s, research to close the tuna life cycle has been conducted to allow the development of tuna aquaculture. In 2002, Japanese scientists from Kindai University closed the life cycle of PBT (Benetti et al., 2016; Sawada et al., 2005). At present, Kindai University produce an average of 80 - 100 t of tuna and between 50,000 - 100,000 juveniles per year (Loew, 2017). Additionally, several Japanese hatcheries have reported an annual production of approximately 60,000 and have projected a full-scale mass production by 2018 with estimates of 200,000 fry per year (Loew, 2017; Van Beijnen, 2017). Additionally, there have been several efforts to close the life-cycle of NBT, and although it has not been as successful as in Japan, in 2014 NBT products originated from these practices became available in different European countries (Van Beijnen, 2017).

1.3 Southern Bluefin Tuna in Australia

In Australia, SBT fishing first started in 1950s, then during 1960s to 1970s there was an increase in the SBT fishing, promoting local economy in Port Lincoln. In 1979, as a consequence of over-exploitation, SBT stocks were becoming limited, pushing fishing vessels to search for tuna further offshore. In 1984, yearly quotas for SBT catching were introduced, leading to the establishment of tuna ranching industry in Port Lincoln, South Australia in 1993, increasing of the value of aquaculture production (ASBTIA, 2015; Nowak, 2004). Today, tuna ranching is considered to be the most valuable sector of aquaculture in South Australia and one of the most important aquaculture industries in Australia, due to its commercial significance (National Aquaculture Council, 2014; Nowak, 2004).

Currently, 2-4 year old SBT juveniles are captured in the Great Australian Bight and transferred to grow-out pontoons for 3-9 months period of fattening on a diet based on frozen and fresh baitfish (Nowak et al., 2006; Valdenegro-Vega et al., 2013). The tuna industry has quickly grown, 8 companies ranch SBT producing in 2017, 8,100 t of gilled and gutted SBT worth AUD$115 million (ASBTIA, personal communication). This species is mainly sold to Japan as sashimi, and even though its price has decreased over time, it is still higher compared to the prices of other fish species (CCSBT, 2018; FAO, 2011).

Adult tunas are relatively unaffected by most pathogens (Munday et al., 2003). Disease outbreaks and health issues in SBT can often be a consequence of an increased pressure of ranching activities (Nowak, 2004). Environmental conditions such as poor water quality, the presence of parasitic hosts (wild fish or intermediate hosts) near the ranching leases as well as, the presence of predators can increase the risk of diseases (Nowak, 2004). The main parasites that can affect ranched SBT are the blood flukes from the genus *Cardicola*, which are considered a serious threat to ranched Bluefin Tuna (Aiken et al., 2009a; Cribb et al., 2011; Polinski et al., 2013), and marine Scuticociliates which have been reported to cause swimmer syndrome (Munday et al., 1997).

1.4 Main blood fluke species affecting farmed and ranched fish

Fish blood flukes, unlike other digeneans, have only one intermediate host, an invertebrate, and a definitive host which is the fish (Bullard and Overstreet, 2002; Cribb et al., 2011). Intermediate and definitive hosts are infected by blood flukes when free living stages (miracidia and cercaria, respectively) penetrate them, therefore, the ability of fish blood flukes to complete their life cycle depends on having both hosts

physically close to each other, so the infective free-swimming life stages can easily reach their hosts (Bullard and Overstreet, 2002; Shirakashi et al., 2016). They infect a wide range of fish, adults are commonly found in the heart and branchial vessels where they can clog the arteries reducing the oxygen exchange causing gill damage and hypoxia. Blood flukes are either host-specific or can have a wide range of fish hosts. Usually, when flukes infect more than one host it is within the same family or genus (Bullard and Overstreet, 2002).

In North America, infections by *Sanguinicola* spp. have caused severe damage to fisheries (Davis et al., 1961; Hoffman et al., 1985). Mass mortalities of Amberjack, *Seriola dumerili,* have been associated with *Paradeontacylix* spp. and *Psettarium* sp. has been found in Japan in Tiger Puffer, *Takifugu rubripes*, imported from China (Ogawa et al., 2007; Ogawa and Fukudome, 1994). In Cutthroat Trout *Salmo clarki* and Columbia River Redband Trout, *Oncorhynchus mykiss*, adults and eggs of the *S. davisi* were found in the gill arches, while embryos were present in gills, kidneys, spleen and heart (Davis et al., 1961). *S. fontinalis* sp. miracidia were infecting the gills of Brook Trout *Savelinus fontalis*, sporocysts and cercariae in the hepatopancreas, while adults were present in the heart and blood vessels (Hoffman et al., 1985). Eggs of *Paradeontacylix* spp. were lodged in the afferent branchial artery and in the ventral aorta and adults in the lumen of the branchial arteries of Amberjack (Ogawa and Fukudome, 1994), and in Tiger Puffer, several haematopoietic organs, gonads, intestine, heart and gills, contained eggs of *Psettarium* sp., while adult flukes were also present in the visceral vascular system (Ogawa et al., 2007).

Species from genus *Cardicola* were initially discovered in 1951 in white trout *Cynoscion arenarius*, and described later by Robert Short in 1952, when they appeared again in Speckled Trout, *C. nebulosus* (Short, 1953). This genus has a

worldwide distribution and some species are considered to be an important pathogen in Bluefin Tuna, causing a big impact on tuna industry (Aiken et al., 2007; Ogawa et al., 2011; Polinski et al., 2014a; Shirakashi et al., 2013). *Cardicola* has an indirect life cycle consisting of two reproductive stages including asexual reproduction which takes place in an intermediate host, a terebellid polychaete and, sexual reproduction in the definitive host, the Bluefin Tuna (Cribb et al., 2011; Ogawa et al., 2017; Shirakashi et al., 2016). To date, four *Cardicola* species (four morphologically described) have been found affecting Bluefin Tuna. *C. opisthorchis* affecting PBT and NBT (Ogawa et al., 2011; Palacios-Abella et al., 2015; Polinski et al., 2013; Shirakashi et al., 2013), *C. orientalis*, present in PBT, NBT, SBT (Neumann, 2017; Neumann et al., 2018; Ogawa et al., 2010; Palacios-Abella et al., 2015; Polinski et al., 2013; Shirakashi et al., 2013) and *C. forsteri*, found in PBT, SBT and NBT (Ogawa et al., 2017; Palacios-Abella et al., 2015; Polinski et al., 2013; Shirakashi et al., 2013). A recent study identified eggs of a yet to be named fourth *Cardicola* species in NBT (Palacios-Abella et al., 2015) (Table 1.2).

Table 1.2. Summary of affected organs of Bluefin Tuna species by *Cardicola.*

Bluefin Tuna species	*Cardicola* species	Affected organ in final host	Reference
PBT	*C. opisthorchis*	Heart	Ogawa et al., 2011
	C. orientalis	Heart and gills	Ogawa et al., 2010
	C. forsteri	Heart and gills	Shirakashi et al., 2016
NBT	*Cardicola* sp.	Unknown	Palacios-Abella et al., 2015
	C. opisthorchis	Heart	Mladineo and Tudor, 2004
	C. orientalis	Heart and gills	
	C. forsteri	Heart and gills	Bullard et al., 2004
SBT	*C. orientalis*	Gills	Shirakashi et al., 2013
	C. forsteri	Heart and gills	Cribb et al., 2000

1.5 *Cardicola*

C. forsteri was identified from the heart of SBT and described by Cribb et al. (2000). The life cycle of *C. forsteri* requires two different hosts, a marine terebellid polychaete *Longicarpus modestus* as an intermediate host in Australia and in Japan, *Neoamphitrite vigintipes* and a definitive host, Bluefin Tuna– SBT, PBT and NBT (Cribb et al., 2011; Shirakashi et al., 2016). The free swimming miracidium enters the intermediate host, in which it undergoes asexual reproduction producing sporocysts. Sporocysts develop into cercariae, a second infective free-swimming stage which infects the definitive host, Bluefin Tuna and where adults can be found and sexual reproduction takes place (Bullard and Overstreet, 2002; Cribb et al., 2011; Kirchhoff et al., 2012; Ogawa et al., 2017; Shirakashi et al., 2016) (Figure 1.1). *Cardicola* cercariae have a distinctive morphology, presenting a well-developed oral sucker (penetrating organ) and a cylindrical body (Cribb et al., 2011; Ogawa et al., 2017; Shirakashi et al., 2016). *C. forsteri* has a long straight tail with no bifurcation (Cribb et al., 2011; Shirakashi et al., 2016), while, *C. orientalis* has a small ellipsoidal tail (Shirakashi et al., 2016). They are poor swimmers, so *Cardicola* life cycle depends on the proximity between intermediate hosts and definitive hosts (Shirakashi et al., 2016). The cercariae infects the host by penetrating directly through the skin, gills and eyes (Aiken et al., 2007; Bullard and Overstreet, 2002; Cribb et al., 2011, 2000; Shirakashi et al., 2013).

C. forsteri adults are found in the heart where they release eggs that will be transported though the circulatory system to the gills, accumulating in the afferent arteries of the filaments (Colquitt et al., 2001). Eggs can also be found in the spongiosa layer of the heart, (Aiken et al., 2009; Colquitt et al., 2001; Cribb et al., 2011; Kirchhoff et al., 2012; Polinski et al., 2014a) where they cause granulomatous reactions and

hypertrophy of the spongiosa, leading to a reduction of the intraluminal area (Colquitt et al., 2001). The eggs travel to the gills through the blood stream, accumulating and obstructing the vasculature of the filament, resulting in the blockage of normal blood flow causing thrombi formation and oedema (Colquitt et al., 2001; Munday et al., 2003). Macroscopic white to yellow lesions in the gills are associated with the presence of eggs, this damage can extend across the gill arch (Colquitt et al., 2001). The clinical signs that have been attributed to *C. forsteri* include lethargy and respiratory distress caused by an increase in gill mucus and branchitis (Munday et al., 2003).

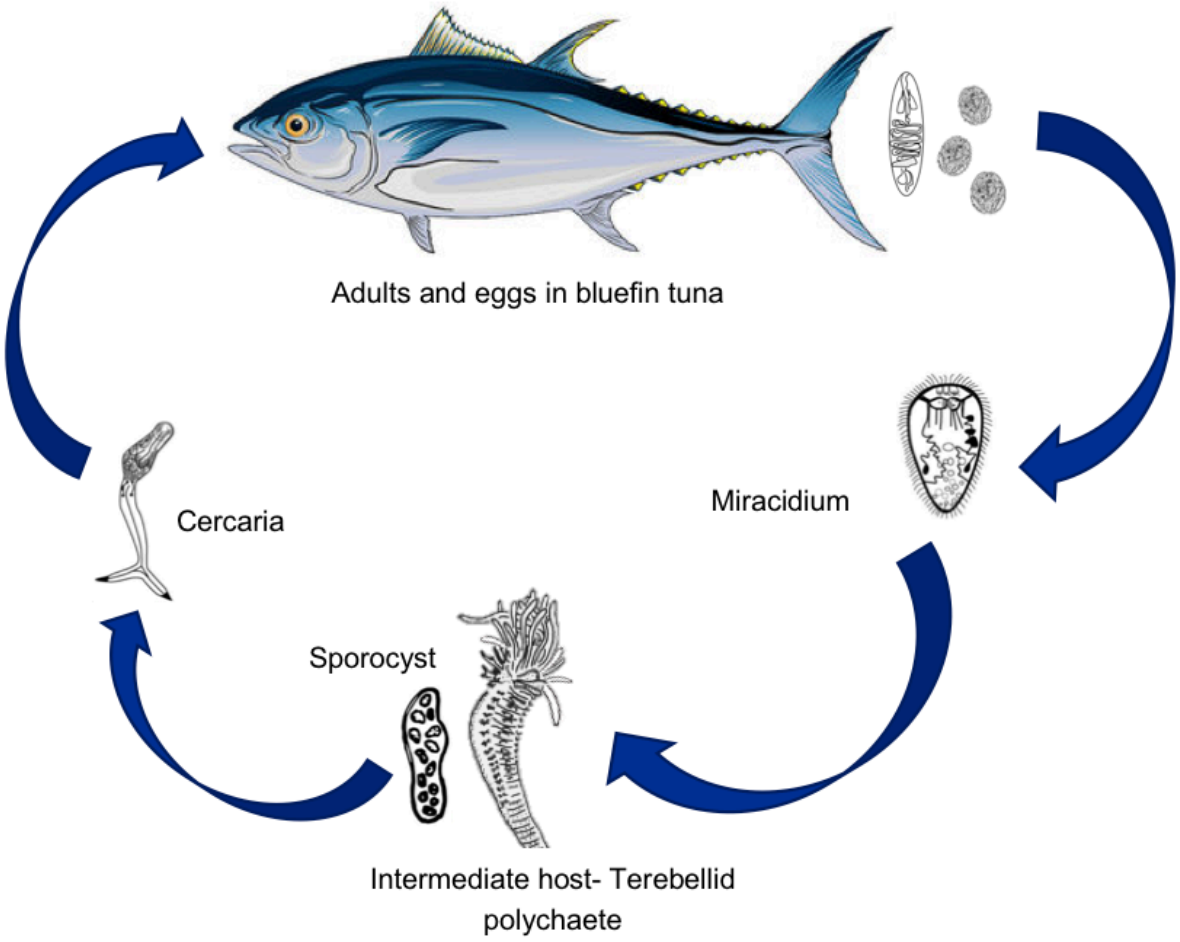


Figure 1.1. Life cycle of *Cardicola*

C. orientalis was discovered in cultured PBT in Japan by Ogawa in 2009. Eggs are mainly present in the gill lamellae and filament arteries, and in the heart muscle but in lower numbers. They vary their shape and size accordingly to the organ where they are found (Ogawa et al., 2010). In SBT, Infections with *C. orientalis* are mainly in the branchial arteries and heart, where adults and eggs can be found, blocking the blood circulation (Polinski et al., 2013; Shirakashi et al., 2013).

Originally described in 2010, *C. orientalis* was first identified based on morphological characteristics and on the partial sequences of the ITS2 rDNA and the 28S subunits, from SBT heart samples in 2012 (Shirakashi et al., 2013), it was detected in serum of ranched SBT in samples from 2008 (Polinski et al., 2013) and in formalin-fixed paraffin-embedding (FFPE) gills and heart ranched SBT samples from 1995, demonstrating the presence of *C. orientalis* in ranched SBT 10 years before being described in PBT (Neumann, 2017). It has been suggested that the presence of *C. orientalis* in SBT was overlooked, as this species shares similarities with *C. forsteri* (Ogawa et al., 2010). Conventional diagnostic techniques, present limitations on differentiation between species, focusing mainly on the detection of adults in heart samples, parasites present in other organs are often missed (Polinski et al., 2013).

The diagnosis of *Cardicola* is based on conventional parasitology methods, using vital organs and relying on the observation and identification by microscopy, of two life stages, adults and eggs present in the heart and gill filaments (Aiken et al., 2006; Munday et al., 2003; Neumann et al., 2018; Shirakashi et al., 2013). Adult flukes can be quantified in heart flushes, identification of the species is possible but problematic as it requires experience (Aiken, 2009b; Neumann et al., 2018). Eggs can be counted directly from the gill filaments but differentiation between *C. forsteri* and *C. orientalis* can be very difficult (Shirakashi et al., 2013). Overall, these methods have

proved to be effective in the diagnosis, but they are time consuming and require experience, they are also limited to the detection of two life stages located in specific organs requiring the destruction of the fish and missing migrating stages which cannot be observed, as well as eggs or adults present in the circulatory system (Polinski et al., 2013).

Conventional diagnosis, as mentioned before, is limited to the detection of only two life stages of *Cardicola* in specific organs, also, differentiation between species is difficult and can be time consuming, therefore, it is necessary to develop new techniques that, using various pathological samples will allow the detection of other life stages and the identification of different species of *Cardicola* (Polinski et al., 2013). Molecular methods such as PCR, have proven to be a reliable tool for the diagnosis of *Cardicola* (Neumann et al., 2018; Polinski et al., 2013). Being highly sensitive and extremely accurate, they also present the advantage of being versatile, allowing the use of non-lethal samples such as serum. They can also be used to detect migrating stages (Neumann et al., 2018; Polinski et al., 2013) and the identification of new species (Palacios-Abella et al., 2015).

1.6 Main Scuticociliate species affecting farmed fish

Scuticociliates have a cosmopolitan distribution, living in the marine sediment and biofouling, grazing on microalgae, bacteria or other protozoa, but under special circumstances, some can behave as opportunistic histophagous parasites, feeding on fish tissues (Moustafa et al., 2010a; Munday et al., 1997). They are considered a serious threat to mariculture around the world (Jee et al., 2001), infecting a wide variety of fish species (Table 1.3). They are responsible for causing mass mortalities in olive flounder *Paralichthys olivaceus* (see Iglesias et al., 2001; Jung et al., 2005;

Moustafa et al., 2010b), turbot *Scophthalmus maximus* (Dyková and Figueras, 1994; Iglesias et al., 2001; Sterud et al., 2000; Whang et al., 2013), sea bass *Dicentrarchus labrax* (see Whang et al., 2013) and Southern Bluefin Tuna *Thunnus maccoyii* (see Munday et al., 1997, 2003).

Scuticociliatosis in olive flounder can be caused either by *Miamiensis avidus, Philasterides dicentrarchi* or *Uronema marinum*, clinical signs include darkening of the skin with haemorrhagic ulcers extending into the underlying muscle and liquefaction of the brain. These Scuticociliates can be present in several tissues and organs such as liver, spleen, kidney, gastrointestinal tract, eyes and skin. *M. avidus* invades the host through skin lacerations or entering the branchial epithelium, from where it can disseminate to the rest of the internal organs (Jung et al., 2007; Moustafa et al., 2010b; Song et al., 2009a). In New Zealand groper, *M. avidus* caused skin lesions leading to an increased mucus production, skin haemorrhages and loss of scales (Salinas et al., 2012).

P. dicentrarchi has been found in sea bass and turbot (Cheung et al., 1980; Iglesias et al., 2001; Jung et al., 2007, 2005). It has been suggested that the entry route could be through the cornea and/or periorbital skin (Iglesias et al., 2001). Based on morphological characteristics and the SSU rRNA sequences, it was proposed that *P. dicentrarchi* and *M. avidus* were synonyms (Jung et al., 2007), but in 2017, a study demonstrated that they are two different species, based on differences in life cycle and morphology, as well as, in the 18S rRNA and the α- and β-tubuline sequences, suggesting that the combination of several techniques such as, morphology, life cycle, multigene analysis and serology, can help in the identification process (De Felipe et al., 2017)

In silver promfret, *Pampus argenteus* (see Azad et al., 2007), *Uronema* sp. infections can result in brown skin lesions, necrosis of the epidermal and dermal musculature, and can also cause clogging of the lumen of the kidney tubules, myositic degeneration of the stomach musculature and liquefaction of the brain. In SBT is has been proposed that initially *Uronema nigricans* parasitises the olfactory rosettes, invading the branches of the olfactory rosette, migrating to the brain causing locomotor dysfunction and the death of the fish (Munday et al., 1997, 2003).

Table 1.3. Examples of Scuticociliates affecting fish, their hosts and the organs they colonize.

Parasite	Scientific name of hosts	Host	Entrance route	Infected organ	Type of microorganism	Reference
Philasterides dicentrarchi	*Paralichthys olivaceus* *Dicentrarchus labrax* *Scophthalmus maximus*	Olive Flounder Sea Bass Turbot	Via small abrasions in skin or gills. Branchial In turbot, though the cornea and/or periorbital skin Gill	Through the skin Haemorrhagic skin ulcers Muscle Branchial Brain Internal organs	Opportunistic	Iglesias et al., 2001 Kim et al., 2004a Moustafa et al., 2010a
Miamiensis avidus	*Paralichthys olivaceus*	Olive Flounder	Directly form sea water. Through skin Gills	Skin Gills Digestive track Liver Kidney Gonads	Opportunistic	Jung et al., 2007, 2005 Song et al., 2009a Moustafa et al., 2010b
Tetrahymena corlissi	*Poecilia reticulate* *Polyprion oxygeneios*	Guppy New Zealand Groper	Immersion through wounded skin lesions	Whitish lesions on the body surface Skin Muscle Viscera Eye socket Spinal cord	Opportunistic	Leibowitz and Zilberg, 2009 Salinas et al., 2012
Pseudocohnilembus persalinus	*Paralichthys olivaceus*	Olive Flounder	Branchial		Opportunistic	Kim et al., 2004b
Uronema nigricans	*Thunnus maccoyii*	Southern Bluefin Tuna	Nasal Olfactory rosettes	Olfactory neuritis Meningitis	Opportunistic	Munday et al., 1997, 2003
Uronema marinum	*Paralichthys olivaceus*	Olive Flounder	Nasal Olfactory rosettes Nerves	Necrotic epidermis Skin Muscle Kidney Urinary bladder Neural canal Gills	Opportunistic	Cheung et al., 1980
Uronema sp.	*Pampus argenteus*	Silver Pomfret	Nasal Olfactory rosettes Nerves	Brownish skin patches Necrotic lesions Distended abdomen Necrotic epithelium of gills Necrotic muscle Gastrointestinal musculature Kidney tubule Necrotic degeneration of brain Olfactory lobes of the brain	Opportunistic	Azad et al., 2007
Pseudocohnilembus longisetus	*Sebastes schlegelii*	Black Rockfish			Non-pathogenic	Whang et al., 2013, 2011

1.7 *Uronema* sp. and *Miamiensis avidus*

Uronema nigricans has been identified as the causative agent of swimmer syndrome in SBT (Munday et al., 1997). Environmental factors, such as the water temperature dropping below 18 ^{o}C have been associated with the development of swimmer syndrome. Initially, the Scuticociliate enters the fish through the olfactory rosettes, colonizing the olfactory nerves and infecting the brain. Fish presented a defective locomotion causing them to energetically swim around the pontoons followed by sinking cycles, eventually the fish sink and dye after failing to continue swimming (Munday et al., 1997, 2003; Nowak, 2007a).

Conventional diagnostic techniques such as staining methods and microscopy, have been widely used in the identification of Scuticociliates (Jung et al., 2011a, 2007; Whang et al., 2013). Morphological characteristics can be useful but unreliable, as Scuticociliates display great similarities, however, when cultivated in vitro they can exhibit significant differences within the same species and isolate, making morphological identification complicated (Jung et al., 2011a; Kim et al., 2004b; Song et al., 2009a; Whang et al., 2013). Additionally, staining techniques can hide differences between individual microorganisms (Budiño et al., 2011; Jung et al., 2011a; Whang et al., 2013). There are only few key morphological differences between *M. avidus* and *U. nigricans* which can generate confusion during the identification process (Jung et al., 2005). Some of the differences are that *M. avidus* does not possess a caudal cilium, the macronucleus is bigger in *U. nigricans*, while the micronucleus is bigger in *M. avidus*, *U. nigricans* is smaller in comparison to *M. avidus* (Table 1.4) (see Jung et al., 2007; Munday et al., 1997; Song et al., 2009a, 2009b). Conventional diagnostic methods along with molecular assays can be helpful identifying Scuticociliates, facilitating the establishment of taxonomical relationships

between species (Jung et al., 2005; Whang et al., 2013, 2011). Molecular identification based on the analysis of the small subunit ribosomal DNA (SSU rDNA) and the subunit 1 of the mitochondrial cytochrome *c* oxidase (*cox1*) sequences, have been successfully used in the identification of these microorganisms (Budiño et al., 2011; Elwood et al., 1985; Hillis and Dixon, 1991; Jung et al., 2005; Whang et al., 2011).

Table 1.4. Morphometric characteristics of *M. avidus* and *U. nigricans*. Data from Jung et al. (2007) and Munday et al. (1997).

Characteristic	*Miamiensis avidus*	*Uronema nigricans*
Body dimensions		
Length (μm)	31.5 (21-37)	26.1 (19-34)
Width (μm)	18.5 (11-28)	11.9 (7.1-20)
Nuclei		
Macronucleus diameter (μm)	6.3 (3.9-6.6)	7.1 (4.2-10.4)
Micronucleus diameter (μm)	1.55 (1.2-2.4)	1.1 (0.6-1.7)
Somatic ciliature		
Total number of kinetics	13 (13-14)	12.4 (12-14)
Length of somatic cilia (μm)	3.55 (2.6-4.8)	3.1 (2.1-5.2)

1.8 Non-lethal sampling

Traditionally, the diagnosis of several diseases affecting fish have been done using lethal sampling (Ek-Huchim et al., 2012). Lethal sampling does not represent a problem when the fish stock is big, but when they are limited by quota, availability or fish are threatened, endangered or expensive, these techniques can become costly or impractical (Cipriano et al., 1996). For the identification of some pathogens,

diagnostic methods requiring lethal samples are considered to be the “gold standard”, nevertheless, they can be impractical and costly (Ek-Huchim et al., 2012). On the other hand, many non-lethal sampling methods have been developed for a wide variety of diseases, proving their capacity to detect a vast range of pathogens (Ek-Huchim et al., 2012; Elliott et al., 2015) (Table 1.5), these non-lethal sampling methods include the use of techniques such as venipuncture, biopsy, gill swabbing, skin swabbing and tissue clips (Altinok et al., 2001; Cornwell et al., 2013; Elliott et al., 2015; Monaghan et al., 2015; Tavares et al., 2016).

Table 1.5. Examples of pathogens that can be diagnosed using lethal sampling and their alternative non-lethal samples.

Pathogen	Fish species	Sample	Non-lethal sample	Reference
Nervous necrosis virus	Atlantic Cod, *Gadus morhua* Atlantic Halibut, *Hippoglossus hippoglossus* L.	Heart, spleen, liver, kidney, muscle. eye, brain, gills, pectoral fin	Blood	Korsnes et al., 2009
Koi herpes	Common Carp, *Cyprinus carpio* L.	Gills, kidney, gut, liver, spleen, brain	Gill mucus, skin mucus, blood	Bergmann and Kempter, 2011 Monaghan et al., 2015
Viral hemorrhagic septicemia	Golden Shiners, *Notemigonus crysoleucas* Fathead Minnows, *Pimephales promelas* Olive Flounder, *Paralichthys olivaceus*	Kidney, spleen	Fin biopsy, gill biopsy	Bain et al., 2010 Cornwell et al., 2013 Hwang et al., 2018
Pancreatic necrosis virus	Atlantic Halibut, *Hippoglossus hippoglossus* L.	Kidney, brain	Blood	Gahlawat et al., 2004 Korsnes et al., 2009
White sturgeon iridovirus	White Sturgeon, *Acipenser transmontanus*	Head tissue	Fin tissue	Drennan et al., 2007
Streptococcus agalactiae	Gilthead Seabream, *Sparus auratus* L. Klunzinger's Mullet, *Liza klunzingeri* Nile Tilapia, *Oreochromis niloticus* L. Red Tilapia, *Oreochromis niloticus* x *O. mossambicus*	Brain, kidney	Kidney aspiration, blood, nasal wash, gill mucus, fecal collection	Evans et al., 2002 Itsaro et al., 2012 Tavares et al., 2016
Yersinia ruckeri	Rainbow Trout, *Oncorhynchus mykiss* Atlantic Salmon, *Salmo salar*	Spleen, liver, kidney	Feces, blood	Altinok et al., 2001 Noga et al., 1988 Ghosh et al., 2018
Renibacterium salmoninarum	Chinook salmon, *Oncorhynchus tshawytscha*	Kidney	Blood, gill filament removal, kidney biopsies, ovary fluid	Elliott et al., 2015 Faisal et al., 2010 Murray et al., 2012
Cichlidogyrus spp.	Nile tilapia, *Oreochrimis niloticus* L.	Gills collected post mortem	Gill mucus	Ek-Huchim et al., 2012 Rahmouni et al., 2017

1.9 Aims of the project

Loss of high value animals from lethal sampling in addition to the potential economic impact parasitic diseases have on ranched Bluefin Tuna is evidence of the need to develop and evaluate non-lethal sampling techniques for Southern Bluefin Tuna. Non-lethal sampling combined with the high sensitivity of molecular techniques can be used for diagnoses or to identify the presence of *Cardicola* and *Miamiensis avidus* near tuna pontoons, including the free-swimming stages of *Cardicola*. Therefore, the aims of this thesis were to:

1. Compare different lethal and non-lethal sampling methods used with conventional and molecular diagnoses for the detection of *C. forsteri* and *C. orientalis*
2. Determine the presence and identify Scuticociliates in samples from SBT showing swimmer syndrome
3. Determine the presence of *C. forsteri* and *C. orientalis* in biofouling samples near SBT pontoons
4. Identify the potential relationship between the substrate, the age of the biofouling sample, type of biofouling organisms and depth, with the presence of *C. forsteri*, and *C. orientalis* DNA in the biofouling

Chapter two

Diseases in Tuna Aquaculture

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Chapter three

Use of non-lethal Southern Bluefin Tuna (SBT) samples for the detection of *Cardicola forsteri* and *C. orientalis* using real-time qPCR

3.1 Introduction

Traditionally, observations of characteristic gross lesions were considered to be enough to make a presumptive diagnosis of blood fluke infection in SBT (Colquitt et al., 2001). The definitive diagnosis relied on conventional methods such as the detection and identification of the adult parasite in heart flushes using a dissection microscope (Aiken et al., 2006; Munday et al., 2003), and the presence of eggs in gill filaments (Kirchhoff et al., 2012; Shirakashi et al., 2012b, 2012a). Histopathology also allowed quantification of eggs in both heart and gills, and observations of adults if present in the section (Aiken et al., 2006; Norte dos Santos et al., 2012).

These methods have proven to be effective in the diagnosis of infections with blood flukes, but they are time consuming and require euthanising the fish so vital organs can be collected (Forte-Gil et al., 2016; Palacios-Abella et al., 2015; Polinski et al., 2013). Overall, they were limited to the detection of either adults or eggs in specific organs, such as heart or gills, missing migrating stages as well as eggs or adults present in the blood vessels (Polinski et al., 2013).

With the development of molecular tools such as real-time qPCR, species-specific diagnosis and quantification are more specific and sensitive (Bell and Ranford-Cartwright, 2002; Johnson et al., 2005; Vetter et al., 2006). PCR has been previously used in the identification of different species from the genus *Cardicola* (Ogawa et al., 2011, 2007; Palacios-Abella et al., 2015; Polinski et al., 2014a, 2013; Shirakashi et al., 2013; Yong et al., 2016), the preferred target being the second internal transcribed spacer of the nuclear rDNA (ITS-2 rDNA) (Ogawa et al., 2011, 2007; Palacios-Abella et al., 2015;

Polinski et al., 2013), combined with the amplification of the subunit I of the mitochondrial cytochrome *c* oxidase gene (*cox*1) (Palacios-Abella et al., 2015) or the 28S region (Ogawa et al., 2011; Shirakashi et al., 2016; Sugihara et al., 2014).

Molecular techniques have proven to be a powerful tool, helping identify the intermediate hosts of *Cardicola* in Bluefin Tuna. The terebellid *Longicarpus modestus* has been described as the intermediate host of *C. forsteri* in Australia and *Neoamphitrite vigintipes* in Japan (Cribb et al., 2011; Shirakashi et al., 2016).. Molecular analysis demonstrated the presence of *C. orientalis* sporocysts in another terebellid, *Nicolea gracilibranchis* (Shirakashi et al., 2016) and larval stages of *C. opisthorchis* were found in *Terebella* sp. (Sugihara et al., 2014).

Fish blood flukes are widely distributed having an important impact on the tuna industry. Molecular tools have been successfully used to study the global distribution of *Cardicola*, annual variability, as well as, the detection of infected intermediate hosts and their distribution near the tuna pontoons (Aiken et al., 2007; Forte-Gil et al., 2016; Neumann et al., 2018; Shirakashi et al., 2017). The presence of *N. gracilibranchis* (intermediate host) infected with *C. orientalis* depends on different factors such as biofouling substrate, often being found on ropes near the pontoons and water depth, for example *N. gracilibranchis* is less abundant in deeper water near the surroundings of the farming pontoons in Japan (Shirakashi et al., 2017).

Molecular techniques such as PCR can also be used to evaluate the presence of *Cardicola* in the definitive host, being capable of detecting migrating life stages such as cercariae or miracidia within the host as well as,

identifying different species and its prevalence in different organs of the host during infection (Neumann et al., 2018; Polinski et al., 2013; Shirakashi et al., 2012b). These techniques can help establish the origin and source of infection as well as monitor infection levels in ranched tuna helping to determine better management plans (Aiken et al., 2009; Forte-Gil et al., 2016; Shirakashi et al., 2012b).

In previous studies, all samples used for conventional and molecular diagnosis of blood fluke infection in tuna involved killing the fish. The conventional diagnosis implies the use of flushes of vital organs such as heart and gills. Using this type of samples makes monitoring of the infection difficult as the individuals have to be killed to collect the samples (Polinski et al., 2013). Hearts need to be examined to recover and quantify adult flukes in the heart flushes (Colquitt et al., 2001; Cribb et al., 2000) and gills have to be dissected to determine the morphology, location and number of eggs in each gill filament (Norte dos Santos et al., 2012; Shirakashi et al., 2012b). Histological analysis of hearts and gills is limited to the identification of granulomas and eggs in stained organ samples, as it cannot identify between *Cardicola* species (Neumann et al., 2018; Norte dos Santos et al., 2012; Yong et al., 2016).

Heart and gill samples have been frequently used to identify the presence of *Cardicola* DNA, requiring the use of lethal sampling for the molecular diagnosis (Neumann et al., 2018; Polinski et al., 2013; Shirakashi et al., 2012b; Yong et al., 2016). The use of gill biopsies has been studied in Atlantic salmon to monitor histological changes in gills avoiding lethal sampling. Results showed that, with the exception of large lesions, biopsy results underestimated the damage in the filaments, as biopsies are a small

representation of whole filaments, offering a limited fragment of the organ with spatial limitations (Nowak and Lucas, 1997).

So far, apart from serum samples, there has been no attempt of using other non-lethal samples for *Cardicola* diagnosis even though molecular diagnostic techniques allow the use of non-lethal samples (Forte-Gil et al., 2016). The use of non-lethal samples helps monitoring the health of the ranched fish as well as, the progress of the infection in individual fish with blood flukes without the need to sacrifice the fish, reducing economical losses sampling causes (Kirchhoff et al., 2012; Polinski et al., 2013).

Being minimally invasive compared to lethal sampling, non-lethal methods can be used for the detection of low infective levels of organisms, working as an effective tool for diagnosis and surveillance (Cornwell et al., 2013; Tavares et al., 2016). They allow sampling of different anatomical parts of one fish and the same individual can be sampled on multiple occasions or during different periods of time (Cornwell et al., 2013; Ek-Huchim et al., 2012), and they have less impact on the fish welfare by reducing the number of sacrificed fish, lowering economical losses for industry, especially when fish are of high value (Cornwell et al., 2013; Tavares et al., 2016) (Table 3.1).

Some of the most commonly used non-lethal sampling techniques includes nips and biopsies which can be taken from gills and fins (Cornwell et al., 2013; Elliott et al., 2015), skin and gill mucus swabs (Bergmann and Kempter, 2011; Cipriano et al., 1996; Ek-Huchim et al., 2012; Elliott et al., 2015; Núñez et al., 2004) and blood sampled through venipuncture (Altinok et al., 2001; Gahlawat et al., 2004; Tavares et al., 2016). Other methods such as kidney aspiration (Noga et al., 1988; Tavares et al., 2016), nasal wash and fecal

collection can also be employed, but these sampling techniques require experience and can potentially cause stress to the fish increase the risk of secondary infections (Tavares et al., 2016).

Table 3.1. Examples of commonly used non-lethal sampling in the diagnosis of different fish diseases.

Method	Species	Target	Pathogen	Reference
Biopsies	Golden shiners Fathead minnows	Gill Fin	viral haemoragic septicaemia virus	Cornwell et al., 2013
Mucus Swab	Koi fish Carp Salmon Chinook salmon Nile tilapia	Gill mucus Skin mucus	Herpes virus	Monaghan et al., 2015
			Aeromonas salmonicida	Bergmann and Kempter, 2011
			Renibacterium salmoninarum	Cipriano et al., 1996
			Cichlidogyrus spp.	Elliott et al., 2015
			Tenacibaculum maritimum	Ek-Huchim et al., 2012
				Núñez et al., 2004
Nips	Chinook salmon	Gill Fin	*Renibacterium salmoninarum*	Elliott et al., 2015
Paper-hole punch	White sturgeon	Pectoral fins	Iridovirus	Drennan et al., 2007
Venipuncture	Atlantic halibut Rainbow trout Nile tilapia	Blood	Pancreatic necrosis virus	Gahlawat et al., 2004
			Yersinia ruckeri	Altinok et al., 2001
			Streptococcus agalactie	Tavares et al., 2016

When combined with molecular diagnostic techniques, non-lethal sampling is equally or more sensitive than conventional diagnosis and traditional sampling methods, identifying the presence of the pathogen in different organs being capable of detecting low infective levels and allowing the identification of possible entry routes (Cornwell et al., 2013; Ek-Huchim et al.,

2012; Tavares et al., 2016). Rapid tests based on molecular techniques along with non-lethal samples have been employed for monitoring the presence of pathogenic bacteria in marine fish, these diagnostic methods allow the detection of pathogenic species, even in asymptomatic fish (Núñez et al., 2004). With methods such as qPCR, positive fish can be identified before the appearance of signs of disease or in the case of bacterial infections, before the bacteria can be cultured, making the detection of the microorganisms during pre-infection phase and in some cases identifying possible carriers (Altinok et al., 2001; Drennan et al., 2007).

The aim of this research was to compare different lethal and non-lethal sampling methods used in conventional and molecular diagnosis for the detection of *C. forsteri* and *C. orientalis* in Southern Bluefin Tuna.

3.2 Materials and methods

3.2.1 Field collection and processing of Southern Bluefin Tuna

Samples were collected in Port Lincoln, South Australia (34°43'56"S 135°51'31"E) from mortalities and harvested ranched SBT during 2015, 2016 and 2017 ranching season, with capture as previously described (Kirchhoff et al., 2011c). To evaluate sublethal testing, sublethal and lethal samples were taken from the same individuals. Heart, gills and gill swabs were collected from individual SBT of approximately 2-3 years of age. Blood was collected from the severed artery in the pectoral recess, using 10 mL non-heparinised collection tubes (Sarstedt, SA, Australia).

Hearts and gills were removed from the fish; hearts were collected and placed in plastic containers. The first and second left gill arch were removed from the fish and sealed in waterproof plastic bags. All samples were held for approximately two hours on ice during the transit from the tuna lease site to the laboratory. Three different types of samples were taken from the gill arches. Gill snips, which are gill clippings were taken from the basal region of the lateral area of the gill arches, individual gill filaments were taken from the distal and middle region of the lateral area. Gill biopsies were taken from individual gill filaments, where gill filaments were divided in three pieces as described by Nowak and Lucas, 1997 (Figure 3.1).

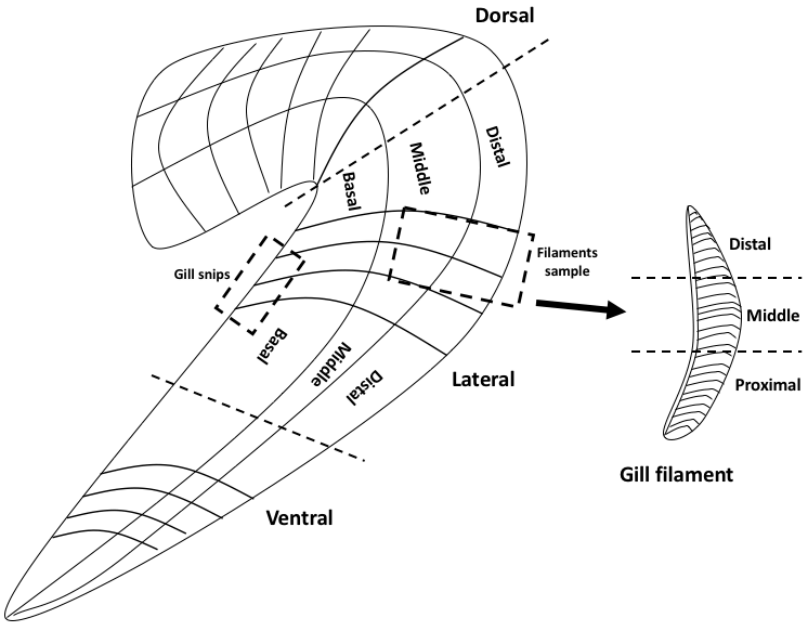


Figure 3.1. Diagram of the gill arch and gill filament, showing the location of the samples collected.

In 2017, three gill mucus samples per fish were collected from 32 SBT, by gentle gill swabbing across the left gill arches using cotton wool buds (Pacific

Laboratory Products) and placed into 1.5 mL plastic tubes with 700 μL of lysis buffer, as a non-lethal sampling method. Gill and swab samples were placed in individual tubes and stored on ice until further analysis (Table 3.2).

Table 3.2. SBT lethal and non-lethal samples collected from 2015- 2017.

Sample	Total samples	Technique	Target
Heart	172	Heart flushes	Adults
Gill snips	30	Real time qPCR	DNA
Gill biopsies	18	Real time qPCR	DNA
Gill filaments	228	Egg counts Real time qPCR	Egg number DNA
Gill swabs	32 (3 per fish)	Real time qPCR	DNA
Serum	172	Real time qPCR	DNA

3.2.2 Laboratory processing

Blood samples were stored at 4^{o}C for 24 hours then centrifuged at 1000 Xg at 4^{o}C for five minutes, serum aliquots were transferred into three 0.6 mL plastic tubes and stored at -20^{o}C.

To detect the presence of adult *C. forsteri*, hearts were dissected through a vertical cut, opening the heart in half though the bulbous arteriosus, ventricle and atrium then flushing with a half-half mixture of sea water and fresh water; the flushes were poured into Petri dishes and examined using a dissection microscope after the red bloods cells had settled.

3.2.3 Egg counts in single filaments

The number of blood fluke eggs in individual filaments was determined using a bright-field microscope with the 10X objective. Briefly, the second left gill arch was rinsed with tap water, removing all the mucus. Gill filaments of approximately 40-60 mm were cut above the gill arch. Individual filaments were selected from each arch and mounted in a wet preparation with a 50/50 mixture of filtered seawater and tap water, placing the filaments between two glass slides. In 2015 six filaments per individual fish were examined, in 2016 and 2017 eggs were counted in four to six filaments for each individual fish (Shirakashi et al., 2012a).

3.2.4 Preservation of gill and swabs samples for molecular analysis

After the eggs were counted in the gill filaments, water excess was dried from each filament by gentile dabbing the sample and each filament was preserved in five volumes of nucleic acid preservation solution (NAPS: 4 M ammonium sulphate, 25 mM sodium citrate, 10 mM EDTA; pH 5.5) in an individual 1.5 mL plastic tubes. Gill snips and gill biopsies were stored in 1 mL of nucleic acid preservation solution and swabs were placed directly in 500 μl of lysis buffer (7.8 M Urea and 0.5% SDS) supplemented with 20 U proteinase K (Bioline, Taunton, MA USA). All samples were stored at 4^{o}C until its further analysis in the laboratory.

3.2.5 Nucleic acid extraction

Total nucleic acid (TNA) was extracted from 50 μl of serum and 10 mg of the NAPS preserved gill samples as follows: NAPS was removed from the gill samples by gentle dabbing on a clean glass plate, serum and gill samples

were placed in a new 1.5 µl plastic tubes. All mucus swabs and gill and serum samples were digested in 500 µl lysis buffer at 55°C for overnight, vortexing occasionally. Once the samples were completely digested they incubated on ice for 10 minutes, following by the addition of 250 µl of ammonium acetate (7.5 M) following by 20 seconds of vortexing. Samples were centrifuged at 14,000 ×g for 5 min at 4°C. 700 µl of isopropanol was added to the supernatant for precipitating the DNA, tubes were incubated for 20 minutes at room temperature. After the incubation period, the sample was centrifuged at 16,000 xg for 15 min at room temperature. The obtained nucleic acid was rinsed twice with 500 µl ethanol (70%) and resuspended in the case of serum, in 50 µl of buffered water (0.05% Triton™ X-100, 10 mM TRIS pH7), and for the rest of the samples, in 100 µl of buffered water.

3.2.6 Cardicola forsteri and Cardicola orientalis real-time qPCR detection

The primers used for this experiment were previously reported (Neumann et al., 2018; Pennacchi et al., 2016) and target heterogeneous areas of the internal transcribed spacer-2 (ITS-2) region of the rDNA, which is specific for *C. forsteri* or *C. orientalis*, available on GenBank (EF661575 and HQ324226, respectively) (Aiken et al., 2007; Ogawa et al., 2011; Pennacchi et al., 2016; Polinski et al., 2013; Shirakashi et al., 2013) (Table 3.3). These primers are capable of amplifying the ITS-2 sequences specific for *C. forsteri* and *C. orientalis* without cross-species of host DNA amplification.

Table 3.3. Primers and probes used and target site (Neumann et al., 2018).

Target	Accession number	Name	Size of the product	Sequences (5'-3')
C. forsteri (ITS-2 rDNA)	EF661575	Cfor_F Cfor_R Cfor_probe	287 bp	TGATTGCTTGCTTTTTCTCGAT TATCAAAACATCAATCGACATC HEX-CCACGACCTGAGCACAAGCCG-BHQ1
C. orientalis (ITS-2 rDNA)	HQ324226	Cori_F Cori_R Cori_probe	191 bp	TGCTTGCTATTCCTAGATGTTTAC AACAACTATACTAAGCCACAA HEX-CACAAGCCGCTACCACAATTCCACTC-BHQ1

Each PCR reaction was performed in a final volume of 10 µl containing 2 µl of TNA template, 400 nM of each primer, 150 nM TaqMan probe labelled at the 5' end with 6-carboxy-2,4,4,5,7,7-hexachlorofluorescein succinimidyl ester (HEX) and a Black Hole Quencher at the 3' end (BHQ; Bioresearch Technologies, CA, USA), 2 x MyTaq™ of mastermix (Bioline, Taunton, MA USA) and molecular water.

Reactions were carried out in a 96 well plates for analysis on a CFX Connect Real-Time PCR Detection System (Bio-Rad, NSW, Australia). The cycling conditions for both probes were 95ºC for 3 minutes, allowing the activation of the DNA polymerase, followed by 45 cycles of 95ºC for 10 s and 60ºC for 30 s, measuring relative fluorescence at the end of each 60°C extension (Neumann, 2017).

3.2.7 Cardicola forsteri DNA recovery test

To determine if the blood processing methods used for obtaining serum and consequently DNA were adequate, 0.2 ng of DNA extracted from *C. forsteri* adult flukes was added to 10 mL of, three tap water samples, three sea water samples and 30 fresh blood samples, collected in 10 mL standard tubes or in 10 mL Sarstedt Z tubes (Sarstedt, SA, Australia). All samples were processed

following two different methods in triplicates. Firstly, after collection, samples were placed over-night at 4 ^{o}C allowing coagulation of the blood, then samples were spun at 800 xg at 4 ^{o}C for 10 min. For the first method, serum, tap water and sea water samples were collected into a new 2 mL microtube, and frozen at -80 ^{o}C until its further use for DNA extraction. For the second method, tap water and sea water samples were collected into a 5 mL tube, containing 3 mL of NAPS.

3.2.8 Limit of detection (LOD) and limit of quantification (LOQ) of Cardicola forsteri in serum

Negative serum samples (confirmed using qPCR for *C. forsteri* and *C. orientalis*) were used to determine the limit of detection (LOD) and the limit of quantification (LOQ) of the PCR tests. DNA from *C. forsteri* adult flukes was extracted and quantified (Qubit®, Invitrogen, USA, Calif). Eight independent DNA dilutions were used to spike negative serum samples, starting with a concentration of 0.1 ng of DNA. Each PCR was performed using four replicates of the spiked serum.

Limit of detection (LOD) and limit of quantification (LOQ) were determined using eight independent replicated dilutions of the *C. forsteri* blood fluke DNA in negative serum. The LOD is the lowest concentration of an analyte that that has a 95% possibility of being detected (Armbruster and Pry, 2008; International Organization for Standardization, 2009). The LOQ is the minimum concentration of an analyte that can be reliable detected and quantified with a coefficient of variation (CV) of 20% (Armbruster and Pry, 2008).

DNA concentration in each PCR reaction was calculated using the mechanistic model “cm3” (Carr et al., 2012) and the qPCR package in the R software v3.2.2 (R Studio© v0.99.902, RStudio Inc. Boston Massachusetts) (R Development Core Team, 2016; Ritz and Spiess, 2008). All PCR reactions were performed in quadruplicate. Spiked negative serum samples were used to confirm the detection of *C. forsteri* DNA in SBT serum using Real-Time PCR. DNA from adult *C. forsteri* flukes previously analysed was used to spike 100 µl and 50 µl of negative serum. DNA extraction was performed as previously described. Briefly: independent dilutions of positive *C. forsteri* DNA was used to spike negative serum samples, starting with a concentration of 0.1 ng to 1.0 ng of DNA.

3.2.9 Statistical analysis

Pearson correlation tests were used to analyse and compare between the presence of *C. forsteri* and *C. orientalis* in gill filaments and serum samples of 2015, 2016 and 2017, using SPSS software (IBM SPSS Software, NY, USA). To calculate the LOD and LOQ, linear regression analysis was used to determine the relationship between independent dilutions of serum spiked with DNA positive for *C. forsteri* and DNA copy number. The relationship between lethal and non-lethal sampling methods was analysed by correlation analysis.

3.3 Results

3.3.1 Intensity of infection and prevalence of Cardicola forsteri and C. orientalis in SBT samples

Adult flukes were detected in 18 heart flush samples (35% of the samples) and eggs were present in 107 gill filament samples (61%) during 2015 and in 2016 there were no adults detected in the 34 samples analysed but eggs were present in 34 of the analysed gill filament samples (100%) (Table 3.4 and 3.5). In 2017 adult flukes were found in 46 heart flushes (53%) and eggs in 51 gill filaments (62%).

In the gill filaments, *C. forsteri* was detected in 82 samples (77%) in 2015 and in 34 samples (100%) in 2016, while in 2017 it was detected in 80 gill filaments (92%). All serum samples from 2015 and 2016 were negative and only five samples (6% of serum samples analysed) were positive for *C. forsteri* while, one sample (1%) was positive for *C. orientalis* in 2017. During 2016, two of the filament samples (1%) were positive for *C. orientalis* and during 2017 it was detected in seven of the filament samples (8%). Overall, 64 of all heart flush samples (37%) were positive for adult flukes and 30 gill filaments presented egg counts (66%). 196 gill filament samples (86%) were positive for the presence of *C. forsteri* and nine (2%) for *C. orientalis*, while five (2%) of all the serum samples were positive for *C. forsteri* and one (1%) for *C. orientalis* (Table 3.4 and 3.5).

Table 3.4. Prevalence of *C. forsteri* and *C. orientalis* infections using different methods to detect adults heart flushes and eggs in gill, and molecular methods to detect DNA in gill and serum using qPCR results from samples of SBT. Sample size shown in brackets, NS- not sampled.

Sample	Method	Sampling year		
		2015	2016	2017
Heart flushes	Count	35% (51)	0% (34)	53% (87)
Gill snips				
C. forsteri	PCR	100% (30)	NS	NS
C. orientalis	PCR	0% (30)	NS	NS
Gill biopsies				
C. forsteri	PCR	NS	100% (18)	NS
C. orientalis	PCR	NS	0% (18)	NS
Filaments				
Eggs	Count	61% (107)	100% (34)	59% (87)
C. forsteri	PCR	77% (107)	100% (34)	92% (87)
C. orientalis	PCR	0% (107)	6% (34)	8% (87)
Serum				
C. forsteri	PCR	0% (51)	0% (34)	6% (87)
C. orientalis	PCR	0% (51)	0% (34)	1% (87)

Table 3.5. Intensity of infection with *C. forsteri* and *C. orientalis* in SBT heart flushes, eggs counts in gill and in gill filaments and serum using qPCR. Sample size shown in brackets, NS- not sampled, NA- not available.

		Sampling year								
		2015			2016			2017		
Sample	Method	Number of positive	DNA per mg average	Eggs per mg average	Number of positive	DNA per mg average	Eggs per mg average	Number of positive	DNA per mg average	Eggs per mg average
Heart flushes	Count	18 (51)	NA	NA	0 (34)	NA	NA	46 (87)	NA	NA
Gill snips	PCR	30 (30)	6.02	NA	NS	NA	NA	NS	NA	NA
Gill biopsies	PCR	NS	NS	NS	18 (18)	675515.90	7.19	NS	NS	NS
Filaments										
Eggs	Count	65 (107)		0.68	34 (34)		1.85	51 (87)		1.42
C. forsteri	PCR	82 (107)	30170.56		34 (34)	149439.9		80 (87)	319315.677	
C. orientalis	PCR	0 (107)	0		2 (34)	209.33		7 (87)	2906.61	
Serum										
C. forsteri	PCR	0 (51)	0	NA	0 (34)	0	NA	5 (87)	19.08	NA
C. orientalis	PCR	0 (51)	0	NA	0 (34)	0	NA	1 (87	1.24	NA

3.3.2 Intensity of infection and prevalence of Cardicola forsteri and Cardicola orientalis in gill mucus swabs

32 gill mucus swabs from SBT were analysed during 2017. 12 SBT samples were positive for *C. forsteri* and nine for *C. orientalis* (Table 3.6). Overall, from the 32 fish sampled (3 swabs per fish), 38% SBT were positive for *C. forsteri* in gill mucus and 28% for *C. orientalis*.

Table 3.6. Prevalence and number of positive samples for *C. forsteri* and *C. orientalis* in heart flushes, gill egg counts and qPCR analysis of gills, serum and swabs in 2017 SBT samples.

		N= 32	
Sample	Method	Percentage of positive samples	Number of positive samples
Heart	Heart flushes	28%	9
Gills	Egg counts	38%	12
	C. forsteri	100%	32
	C. orientalis	0%	0
Serum	*C. forsteri*	3%	1
	C. orientalis	0%	0
Swabs	*C. forsteri*	38%	12
	C. orientalis	28%	9

3.3.3 Comparison between lethal sampling and non-lethal sampling

Lethal sampling results showed a poor relationship between eggs present in the filaments and adults recovered from heart flushes. During 2015 and 2016, 97% of the gill filament samples presented egg counts, while only 21% of the heart flushes had adult flukes. A comparison between the relative percentages of the results using heart flushes and egg counts during 2017 showed there was little difference between prevalence results for both methods, detecting adults in 53% of the heart flushes and eggs in 59% of the filament samples (Table 3.4), but when results of individual fish were compared, there was a weak negative correlation between number of eggs present in gills and the adults found in the heart of SBT, as fish with adults in the heart did not always have eggs in the gills, therefore, results using these techniques can exhibit discrepancies, resulting in false negatives and underestimating the presence of the parasite (Figure 3.2).

Samples were tested to determine if they have a normal distribution. Wilcoxon Rank Sum Test for Independent Samples was used as a non-parametric test, complementary to the correlation tests presented in this chapter. The results of these tests is included as an appendix at the end of the thesis.

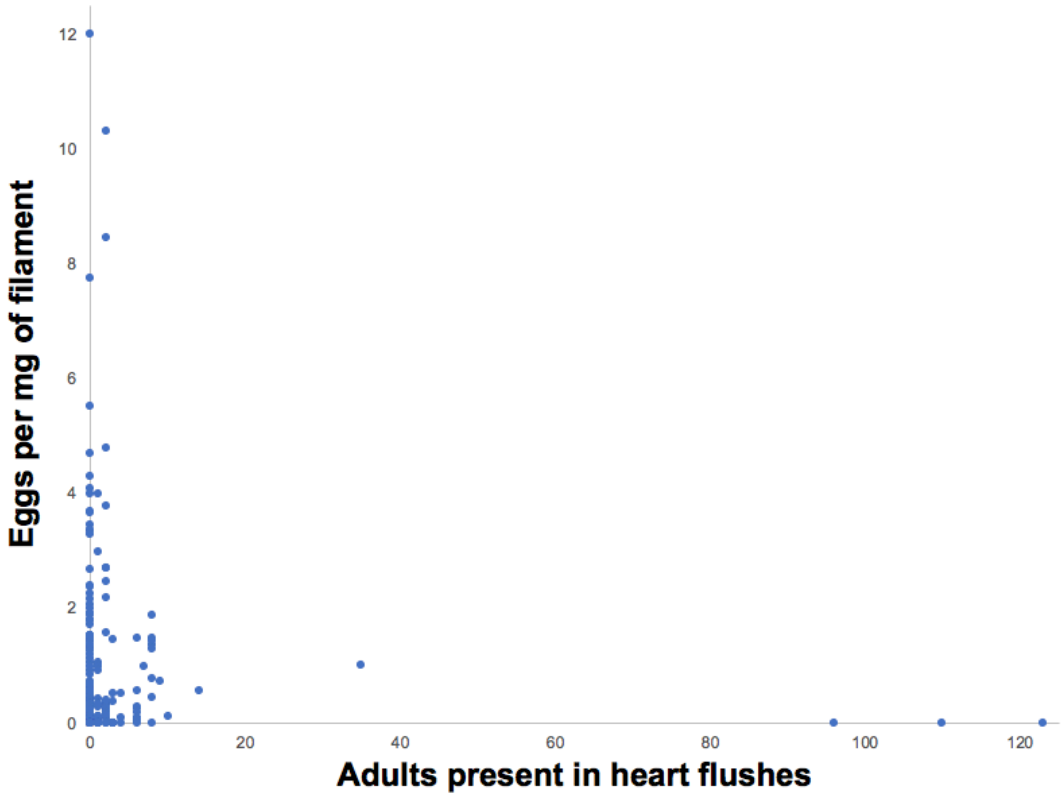
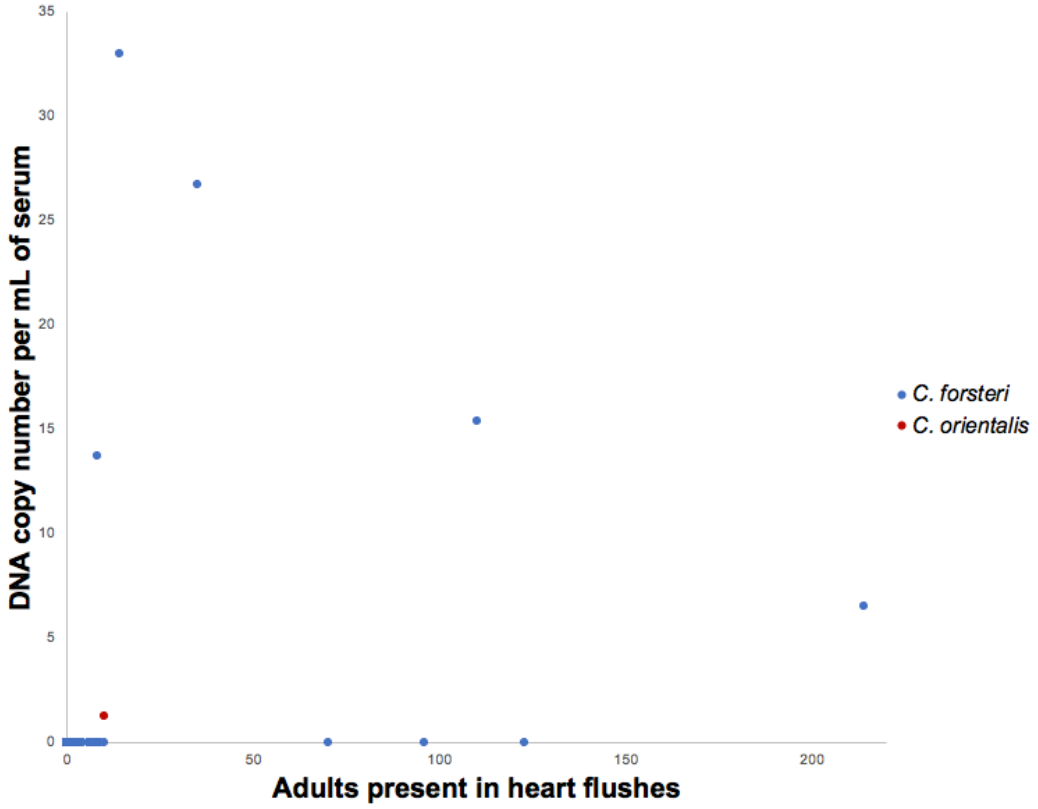


Figure 3.2. Relationship between adult flukes present in heart flushes and eggs detected per mg of gill filament, each dot represents an individual SBT. Samples collected during 2015, 2016 and 2017.

All serum samples from 2015 and 2016 were negative for the presence of *C. forsteri* and *C. orientalis* using qPCR, in 2017 five samples of serum were positive for *C. forsteri* and only one sample was positive for *C. orientalis*. These results were compared against the corresponding adult flukes present in heart flushes and the DNA copy number per mg of gill filaments. Results obtained from analysing adults in the heart and DNA copy number from serum showed that few samples positive for adults in heart were also positive for the detection of *Cardicola* in serum (Figure 3.3), similar to the results obtained from DNA copy number per mg of filament and DNA copy number in serum, where few fish with positive filaments had also positive serum (Figure 3.4). All positive

serum samples were obtained from the same company, from two different pontoons, also, all positive serum samples had blood flukes in the heart and eggs present in the filaments. In the case of two of the samples, the number of adult blood flukes recovered was between 100-200 per SBT. All samples were collected between May and July. The average of adult flukes found in negative serum samples during May was 13.68 per SBT and during July was 2.6 adult flukes per SBT. In contrast, the average of adult flukes found in SBT



with positive serum during May was 25.79 blood flukes and in July was 4.67 (Table 3.7).

Figure 3.3. Relationship between adult blood flukes present in heart flushes and DNA copy number of *C. forsteri* and *C. orientalis* per mL of serum. Each dot represents an individual SBT, samples collected during 2015, 2016 and 2017.

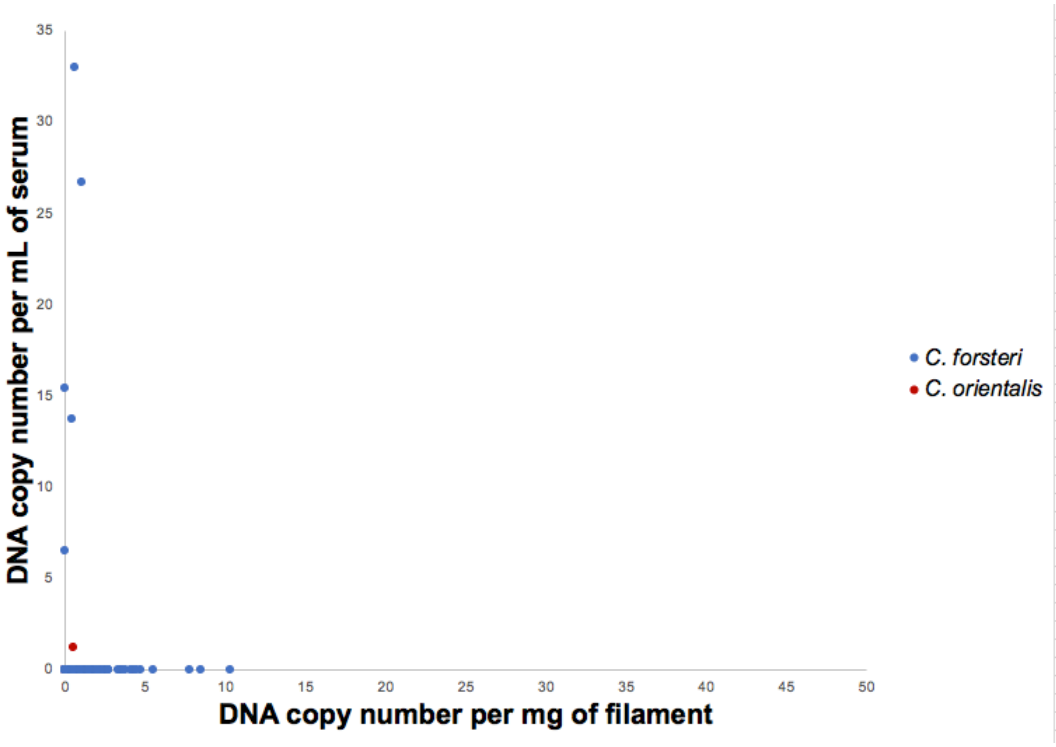


Figure 3.4. Relationship between *C. forsteri* and *C. orientalis* DNA copy number per mg from gill filaments and the DNA copy number per mL from serum sample. Each dot represents an individual SBT, samples collected during 2015, 2016 and 2017.

Table 3.7. *C. forsteri* and *C. orientalis* positive serum samples and number of blood flukes found in each SBT, the time of the year and pontoons where they were collected from. Each row is showing one SBT. ND – not detected.

			Serum DNA copy number per mg		
Month sampled	**Pontoon**	**Adult flukes (heart)**	***C. forsteri***	***C. orientalis***	
May	1	110	15.4	ND	
May	1	214	6.5	ND	
July	2	35	26.7	ND	
July	1	14	33	ND	
July	2	8	13.7	ND	
July	2	3	ND	1.2	
Average adult flukes present in the heart in all serum positive SBT during May					25.79
Average adult flukes present in the heart in all serum positive SBT during July					4.67
Total adult flukes present in the hearts of all samples collected in May					619
Total adult flukes present in the hearts of all samples collected in July					112

Detection of *Cardicola* DNA from gill samples proved to be the most effective method when compared to other non-lethal and lethal sampling techniques. 100% of the gill snips and 36% of the corresponding heart flushes collected during 2015 were positive. Results from 2015, 2016 and 2017 showed that qPCR tests from gill filaments were capable of detecting the presence of *C. forsteri* and *C. orientalis* from fish with no adult flukes in heart and in filaments with no eggs (Figure 3.5 and 3.6).

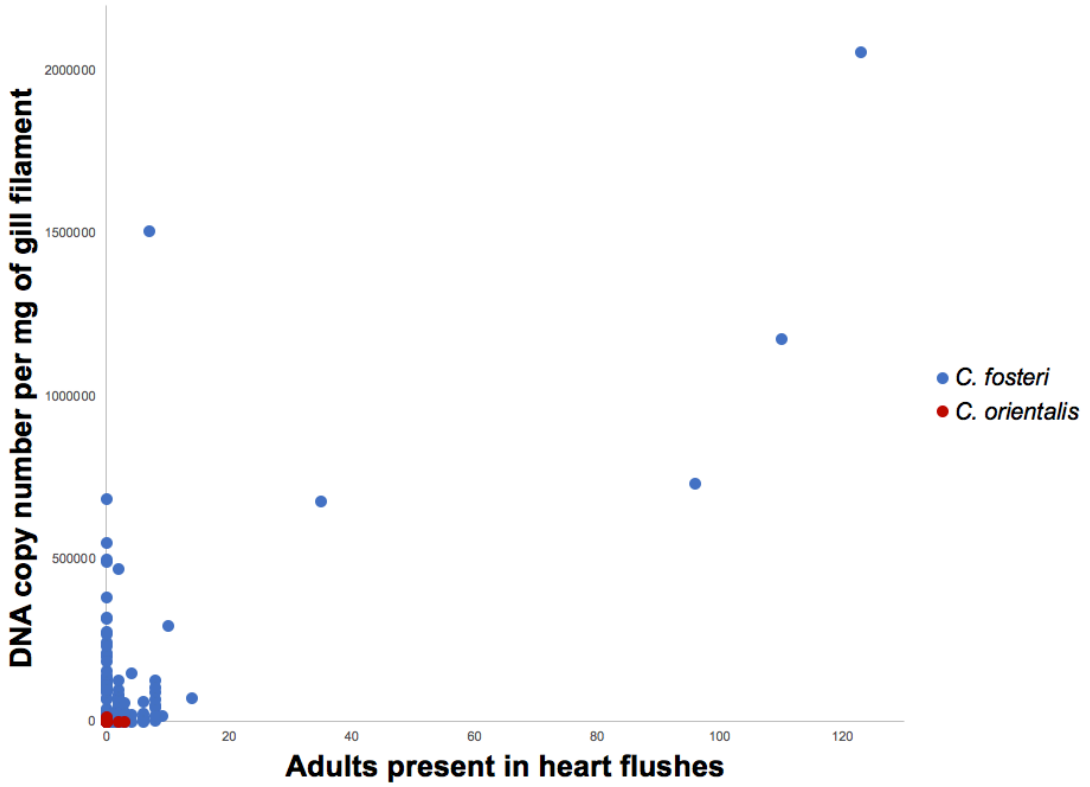


Figure 3.5. Relationship between adult flukes of *C. forsteri* and *C. orientalis* detected in heart flushes and DNA copy number per mg of gill filament in SBT from 2015, 2016 and 2017. Each point represents and individual SBT.

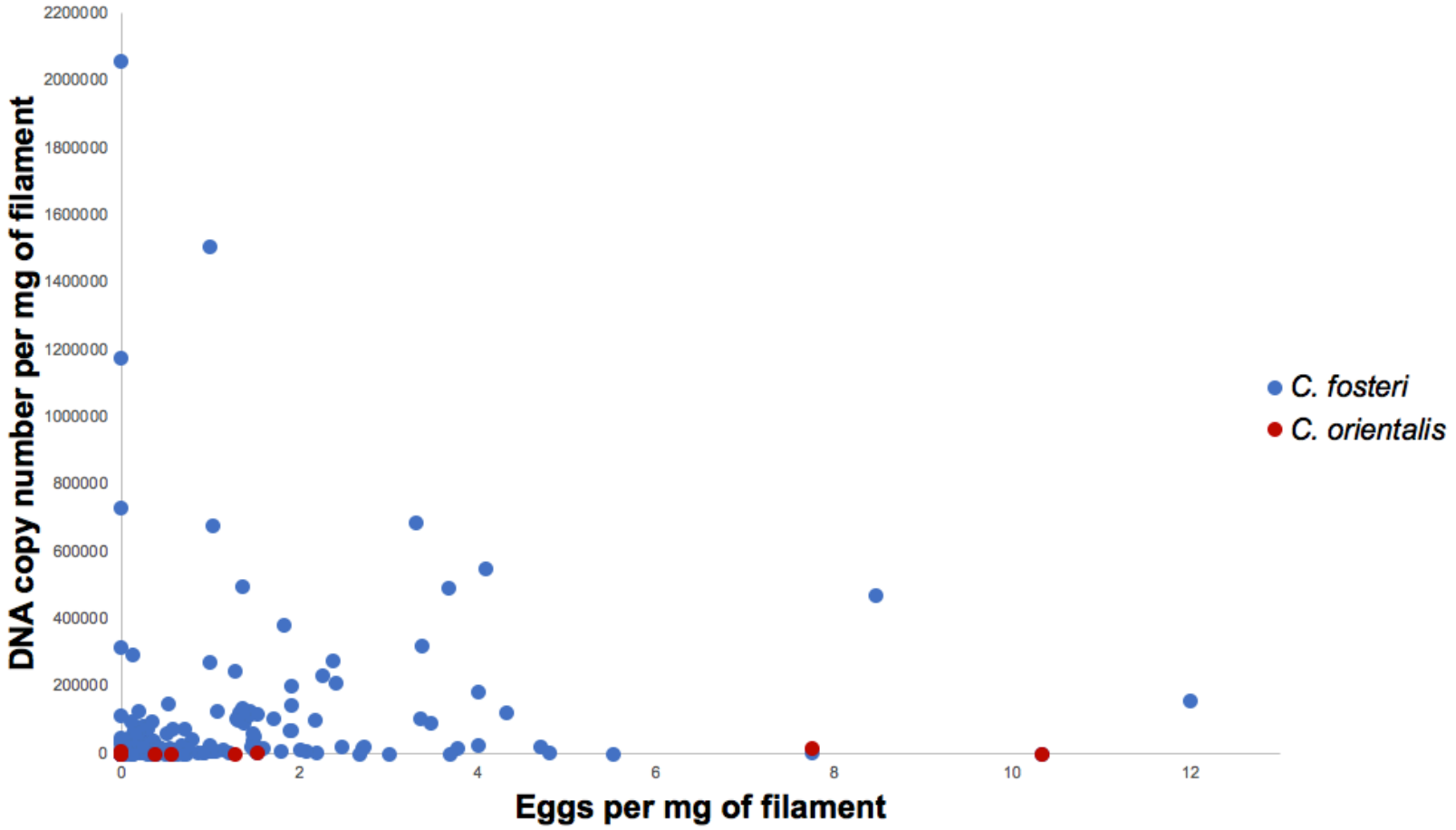


Figure 3.6. Relationship between eggs present per mg of filament and DNA copy number of *C. forsteri* and *C. orientalis* per mg of filament in SBT, each dot represents individual SBT. Samples collected during 2015, 2016 and 2017.

C. forsteri was detected in 100% of gill biopsies taken from filaments with low, between 30-31 eggs per filament, medium 53-55 eggs per filament, and high 102-109 eggs per filament, egg counts. Correlation analysis showed a strong positive correlation between eggs per mg of biopsy and DNA copy number per mg of sample.

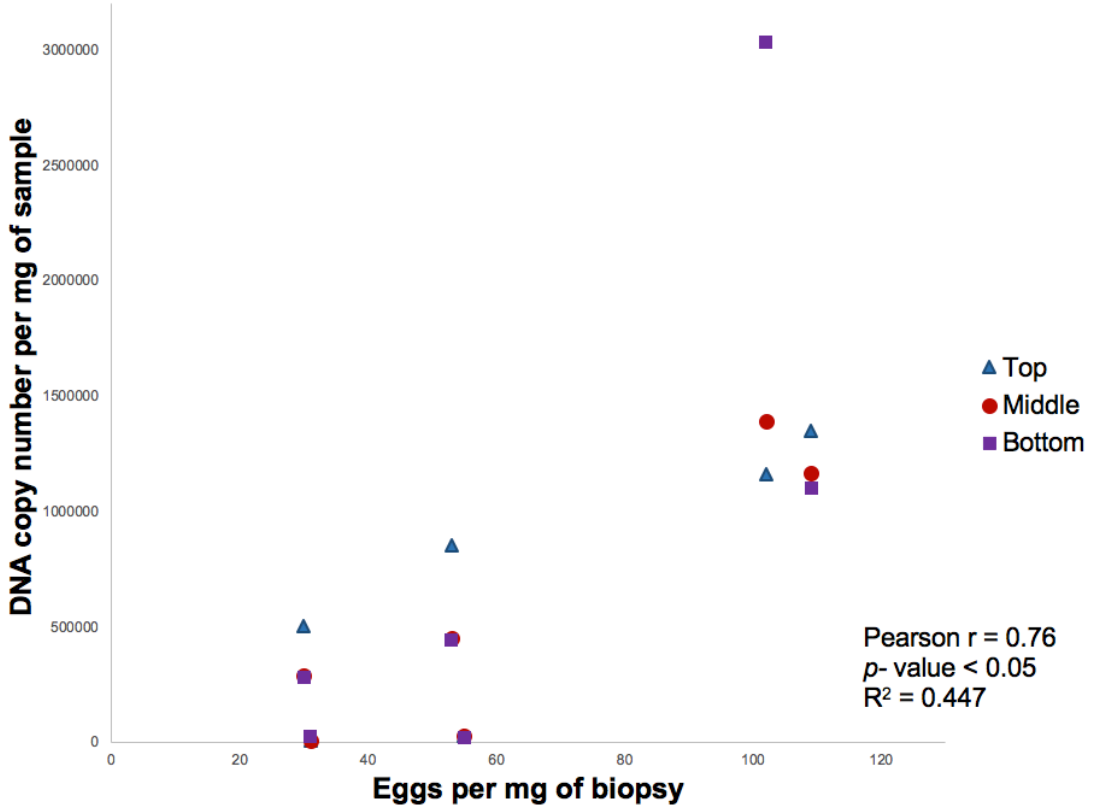


Figure 3.7. Relationship between eggs present per mg of gill biopsy and DNA copy number of *C. forsteri* per mg of biopsy. Four different filaments were used, each filament was divided into three samples: top, middle and bottom. Samples collected in 2016

There was little difference between detecting adults in 47% of the heart flushes and eggs in 50% of the filament samples using qPCR. DNA detection

using qPCR assays from gill filaments proved to be the most effective method, where *C. forsteri* had a prevalence of 92% and *C. orientalis* of only 8%.

C. forsteri and *C. orientalis* were both detected in gill mucus. Eleven (34%) mucus samples were negative for both *C. forsteri* and *C. orientalis*, while 21 (66%) samples were positive for *C. forsteri, C. orientalis* or both. Swabs showed a lot of variation between results from the same fish, as the results for the three swabs were not always consistent. Three mucus samples were positive only in 4 fish (13%) for *C. forsteri* and 5 fish (16%) for *C. orientalis* (Table 3.8). Neither of the samples positive for both species of *Cardicola* were detected in all the three samples at the same time (Table 3.9).

Table 3.8. Number of mucus samples positive for *C. forsteri* and *C. orientalis*, n=32

C. forsteri			*C. orientalis*			
1 swab	2 swabs	3 swabs	1 swab	2 swabs	3 swabs	
19% (6)	6% (2)	13% (4)	6% (2)	6% (2)	16% (5)	
Total negative samples						34% (11)
Total positive samples						66% (21)

Positive gill samples did not show any correlation with the number of adults present in heart flushes, eggs per mg of gill filament or DNA copy number per mg of gill filament. All gill filaments were negative for the presence of *C. orientalis,* while nine mucus samples were positive for *C. orientalis* (Table 3.9).

Table 3.9. Positive gill mucus swabs analysed for *C. forsteri* and *C. orientalis* and their respective number of adults, eggs and DNA copy number per mg of gill filament. Three swabs per fish were collected and processed. Each row represents data for one SBT.

		DNA copy number per mg of gill filament		PCR detection Number of positive mucus swabs	
Number of Adults	Eggs per mg of filament	*C. forsteri*	*C. orientalis*	*C. forsteri*	*C. orientalis*
0	0.29	2741.79	0	2	3
0	0.25	19233.97	0	1	1
1	0.12	9.44	0	0	3
14	0.57	72539.22	0	0	3
0	0.7	47.48	0	1	0
0	0	4723.77	0	0	3
1	0	9.5	0	1	2
1	0	4173.71	0	1	1
2	4.45	863.66	0	1	3
1	0.32	0.92	0	3	0
0	0	316402.2	0	3	0
0	0.2	11884.53	0	3	0
.0	0.08	269402.14	0	3	0
0	1.46	154486.68	0	1	0
0	0.36	9390.72	0	0	2
0	0.47	183573.54	0	2	0

Gill mucus analysis of individual samples showed a reduced capacity to identify positive samples, detecting the presence of *C. forsteri* in 38% of the samples and *C. orientalis* in 28%, the detection of both *C. forsteri* and *C. orientalis* in single samples had a prevalence of 16%. Gill filaments were the only other sample where both *Cardicola* species were detected, with only 10% of positive gill filaments for both species of *Cardicola.* Serum analysis showed poor detection, where only 3% of serum samples analysed were positive for *C. forsteri* and 1% to *C. orientalis* (Figure 3.8).

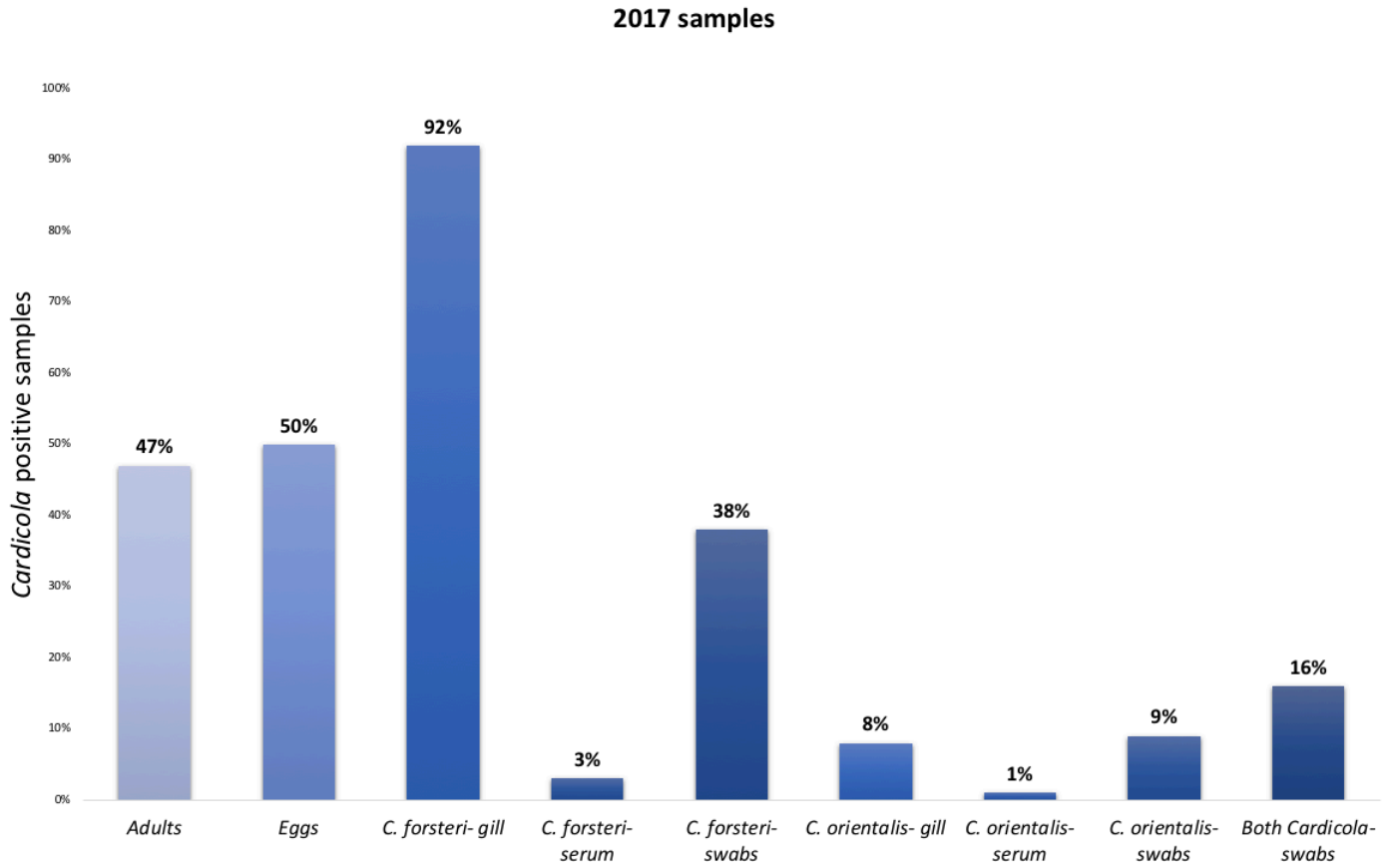


Figure 3.8. Comparison of the relative percentage of positive samples collected during 2017, obtained by conventional methods, adult flukes in heart flushes and egg counts in gill filaments, in contrast to positive gill filaments, gill mucus swabs and serum samples by qPCR, n= 32.

3.3.4 Cardicola forsteri DNA recovery test

The DNA recovery test performed in serum showed that the method used for processing did not interfere with the amount of TNA obtained after the DNA extraction. From 30 serum samples analysed 27 samples were positive, this is 90% positive samples, LOD and LOQ corresponded to the results previously reported (Neumann et al., 2018). All tap water and sea water samples were positive for *C. forsteri*.

3.3.5 Limit of detection (LOD) and limit of quantification (LOQ)

C. forsteri DNA could be detected in all spiked samples. The spiked serum trial showed that the test is capable of detecting 1 single copy of DNA, with a LOD for the assay of 5 copies and a LOQ of 25 copies of DNA (Figure 3.9).

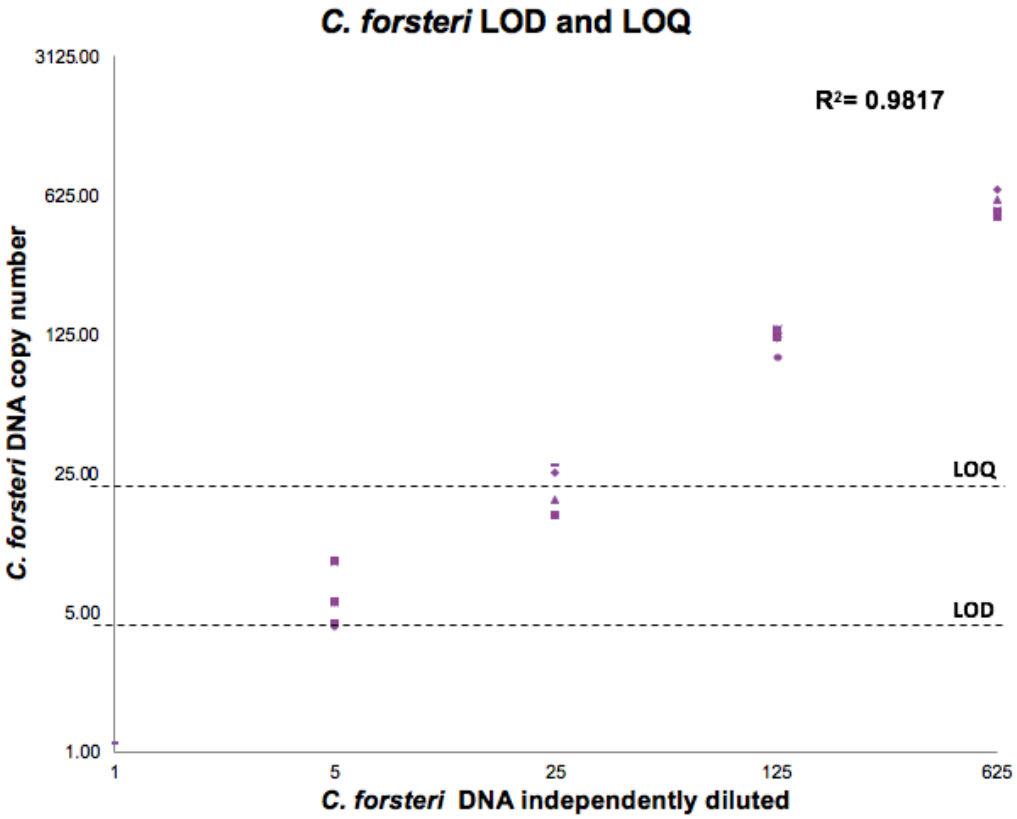


Figure 3.9. Data present the log5 reduction of *C. forsteri* DNA. The limit of detection (LOD) and limit of quantification (LOQ) for *C. forsteri* in serum, real time qPCR assays. Data obtained from eight independent dilutions and performed by quadruplicate. First four dilutions not shown, as results were negative, having less than a DNA copy. DNA concentration of 0.1- 1.0 ng of DNA.

3.4 Discussion

The results of the present study showed the differences between lethal and non-lethal sampling sensitivity in the diagnosis of *C. forsteri* and *C. orientalis* from single individuals. Conventional diagnostic techniques use lethal sampling, relying on the identification of eggs and adults of *Cardicola* using morphology, making it difficult to differentiate between species. These methods are time consuming and require expertise. Molecular techniques were more sensitive, detecting the presence of *Cardicola* in fish considered to be negative by conventional methods, they allowed the

differentiation between species of *Cardicola*, as well as, the detection of life stages which cannot be observed, such as miracidia or cercaria (Polinski et al., 2013). DNA detection using molecular methods such as PCR and qPCR, along with non-lethal sampling have proven to be an effective diagnostic tool, reducing the invasiveness causing minimal damage to the fish, they are highly specific and in some cases, with a sensitivity comparable to conventional methods, it is becoming common in the diagnosis of several diseases in fish (Cornwell et al., 2013). PCR-based assays are capable of differentiating between species, delivering results in less time, compared to conventional diagnostic methodologies.

Variable outcomes using diagnostic techniques requiring lethal sampling methods, have been previously reported for *Cardicola* in SBT (Neumann et al., 2018; Norte dos Santos et al., 2012). The results obtained in this study showed discrepancies between diagnostic results using lethal sampling methods. There is no correlation between the number of eggs observed in gill filaments and adults recovered from heart flushes from the corresponding individual fish. A previous study has also reported lower incidence of adults in the heart when compared to the eggs present in gills (Neumann et al., 2018), as adults release multiple eggs, a strong correlation between adult blood flukes and eggs in gill filaments would not necessarily be expected. Higher concentration of DNA of *Cardicola* in the gills could also mean that there is a higher concentration of *Cardicola* in that organ, as gills are the main route where *Cardicola* enters and exits SBT.

Recovery of adult blood flukes in the heart could also be influenced by the use of anthelmintics such as praziquantel. A previous study demonstrated that the recovery of *Cardicola* DNA in heart and gills of SBT were significantly reduced after praziquantel treatment (Polinski et al., 2014a), and in 2012 Hardy-Smith et al. tested

the efficiency of praziquantel *in vitro*, demonstrating the capacity of this anthelmintic to stop the response of blood flukes; and *in vivo,* finding that the treatment with praziquantel reduced the number of blood flukes in the hearts of SBT. In PBT, it was demonstrated that after administering praziquantel for three consecutive days, dead adults flukes were found after the second day, and were eliminated from the hearts after 11 days, while eggs present in the gill filaments stayed viable after the treatment (Shirakashi et al., 2012b). As ranched SBT were treated with praziquantel, it is possible that the treatment killed the adults but other life stages, such as eggs, were unaffected, thus, fewer adults were observed in the heart, while larger amounts of blood fluke DNA were detected in gills.

Similar to eggs in gill filaments, qPCR results of gill snips did not show correlation with the adults present in the heart, as 100% of the gill snips were positive for *C. forsteri* and only 3% of the corresponding hearts were positive for blood flukes, showing differences in the results obtained between these techniques and resulting in possible false negatives, underestimating the presence of the parasite in the fish by relying in the presence of only one life stage. Although there was no correlation between DNA copy number from gill filaments and adults in the heart, as most of the heart flushes recovered low numbers of blood flukes, a low positive correlation between number of qPCR positive filaments and samples with adult flukes was observed.

Real time qPCR using gill filaments showed that the technique is capable of identifying positive samples, even when they were negative for the presence of eggs, suggesting that it might be detecting other *Cardicola* life stages in the gills, as previously suggested (Polinski et al., 2013). qPCR has proven to be the most sensitive technique, identifying 86% of all filament samples as positive. Having the advantage

of being a rapid quantitative test, allowing the differentiation between *Cardicola* species, it can be easily adapted to field conditions. Special techniques using gill filaments for direct observation, allow the detection and the identification of morphological differences between *Cardicola* eggs, as reported by Shirakashi et al., (2012b), these techniques require expertise and are time consuming and are limited to the detection of only one life stage.

Molecular analysis of gill biopsies showed that the qPCR can detect positive samples with low, medium and high egg counts in SBT samples. Biopsies are considered a non-lethal method allowing the collection of samples from different anatomical parts as well as organs. They have been successfully used for the diagnosis and detection of fish pathogens, for example of Viral hemorrhagic septicemia virus in Golden Shiners, *Notemigonus crysoleucas* and Flathead Minnows, *Pimephales promelas* (Cornwell et al., 2013), *Y. ruckeri* in Rainbow Trout, *Oncorhynchus mykiss* (Noga et al., 1988) *S. agalactiae* in Nile Tilapia, *Oreochromis niloticus* (Tavares et al., 2016) and koi herpesvirus in Common Carp, *Cyprinus carpio* (Monaghan et al., 2015). Nevertheless, in some cases biopsies have shown to be less effective, allowing a poor identification of microorganisms present in tissue samples (Korsnes et al., 2009; Nowak and Lucas, 1997). As the size of a biopsy sample is considerably reduced when compared to samples obtained through lethal sampling, it is important to use a sensitive diagnostic method, such as qPCR. Further studies will need to be performed to identify the best region in the gill for sampling, as well as to determine the number of biopsies required per fish and number of fish that should be sampled.

Swabbing has been reported to be an effective non-lethal sampling method, allowing the detection of different pathogens. It is also a flexible tool, as it can sample

different anatomical areas such as skin and gills, during different periods of time and infection stages (Bergmann and Kempter, 2011), identifying possible transmission routes of the pathogen and allowing an early detection, giving the opportunity to intervene in the infectious cycle without euthanising the fish (Cipriano et al., 1996; Núñez et al., 2004). Along with molecular biology techniques, it has shown great sensitivity being able to detect low infectious levels (Ek-Huchim et al., 2012). Skin swabs have been used in the detection of fish pathogens, for example *Tenacibaculum maritimum* in Turbot, *Scophthalmus maxima* (Núñez et al., 2004) and *Aeromonas salmonicida* in Coho Salmon, *Oncorhynchus tshawytscha* and Chinook Salmon, *O. kisutch* (Cipriano et al., 1996). Gill mucus swabs have been successfully employed for the diagnosis of koi herpesvirus in Common Carp, *Cyprinus carpio* (Bergmann and Kempter, 2011; Monaghan et al., 2015) and Viral hemorrhagic septicemia virus in Rainbow Trout, *Oncorhynchus mykiss* (Cornwell et al., 2013), as well as, in the detection of *Streptococcus agalactiae* in Nile Tilapia, *Oreochromis niloticus* (Tavares et al., 2016).

In this study, swab samples allowed the identification of *C. forsteri* and *C. orientalis* present in SBT gill mucus, even though DNA quantification from mucus samples is possible, it was not attempted in this research as the volume of mucus collected was unknown. The test proved to be useful in demonstrating the presence of different *Cardicola* species and can potentially be as effective as counting eggs in the gill filaments without damaging the fish, it can also be used to monitor the state of health of the fish and help establishing better programs for prevention and control of outbreaks.

As swabbing samples are limited to a small anatomical area, in order to ensure the detection of *Cardicola* in gill mucus, it is required to take at least three samples

per fish, as our results showed that one swab could potentially lead to false negatives. In order to make gill mucus a quantitative technique, it is necessary to know the exact amount of mucus collected, therefore, sampling should be performed using different non- absorbent materials such as rubber spatulas for mucus scarping, that will allow the mucus collection and transfer into recipients such as tubes. Other mucus collection techniques such as mucus aspiration using pipettes, could also be tested.

Venipuncture is another common non-lethal sampling, used in blood tests, vaccine evaluation and measurement of immunological parameters (Cogun et al., 2012; Pasnik et al., 2005). Blood samples can also be used for pathogen detection, for example the diagnosis of pancreatic necrosis virus in Atlantic halibut (Gahlawat et al., 2004) and *Yersinia ruckeri* in rainbow trout (Altinok et al., 2001), *Streptococcus agalactiae* in Nile tilapia (Tavares et al., 2016). Blood sampling has the advantage of being able to sample the same fish over a given period of time without the need to of necropsies (Altinok et al., 2001; Tavares et al., 2016) becoming a potential screening tool with field applications (López-Vázquez et al., 2006). In SBT, serum samples have been used to follow the immune response against *Cardicola forsteri* (Aiken et al., 2008; Kirchhoff et al., 2012; Pennacchi et al., 2016), they have also been employed to monitor and determine the efficiency of anthelmintic treatments, by measuring the levels of praziquantel in blood (Ishimaru et al., 2013) or by correlating praziquantel dosage with the infection intensity, recovering adults in heart flushes samples and observing egg counts in gill filaments (Hardy-Smith et al., 2012; Ishimaru et al., 2013; Shirakashi et al., 2012a) and detect of *C. forsteri and C. orientalis* (Polinski et al., 2013). However, in this study all serum samples collected 2015 and 2016 were negative, only five samples of 2017 were positive for *C. forsteri* and one of the same

year for *C. orientalis,* suggesting that for some reason it was not very sensitive method despite good recovery from DNA spiked blood.

The results obtained in this study have shown that there is a difference between lethal and non-lethal sampling. Non-lethal sampling allows early and rapid detection potential infections (Monaghan et al., 2015). They have been recognized as strong tools which can be successfully combined with molecular diagnostic methods (Cornwell et al., 2013). The efficiency of non-lethal sampling depends on the pathogen entry route, for example, for the diagnosis of *T. maritimum*, skin swabbing is the most adequate method, as the pathogen enters thought the skin mucus (Cornwell et al., 2013; Núñez et al., 2004).

Cardicola is regarded as a serious threat to tuna industry, causing high mortalities, two species of *Cardicola* have been detected in SBT, *C. forsteri* and *C. orientalis*. The diagnosis requires lethal sampling including hearts where the adult flukes can be found and gills to do egg counts. Each Bluefin Tuna has a high commercial value, therefore, using lethal sampling has an important economic impact. The development of non-lethal sampling that allows the monitoring of the state of heath of the fish is important, being an alternative sampling method for surveillance and diagnosis of *Cardicola* in SBT. The use of gill mucus swabs has shown promising results, suggesting that this could be a powerful technique in the detection of *Cardicola*. Further research using this technique in SBT is needed to help standardize this method, allowing better recovery of *Cardicola* present in the gills of Tuna.

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Chapter four

Molecular characterization of *Miamiensis* avidus (Ciliophora: Scuticociliata) from ranched Southern Bluefin Tuna, *Thunnus maccoyii* off Port Lincoln, South Australia

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Published in:

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4.1 Abstract

Scuticociliates are opportunistic protozoan pathogens present in a wide range of teleost hosts. *Uronema* spp. and Miamiensis spp. are the two most common genera recorded from Scuticociliatosis cases in farmed and ornamental fish. Southern Bluefin Tuna (*Thunnus maccoyii*) (SBT) ranching is a high value aquaculture sector, situated off Port Lincoln, South Australia. *Uronema nigricans* has been previously associated with SBT swimmer mortality syndrome and was considered to be the causative agent. We conducted the first molecular characterisation of swimmer syndrome agent from affected SBT. Comparison of SSU rDNA and mitochondrial cytochrome *c* oxidase 1 sequences from the cerebrospinal fluid from SBT affected by swimmer syndrome and *marinum* samples, and phylogenetic analyses identified the Scuticociliate present in SBT samples as *Miamiensis avidus*. Bayesian Inference analyses of both partial gene sequences of the Port Lincoln isolates form a clade with known *M. avidus* to the exclusion of Uronema spp. This shows that *M. avidus* is associated with swimmer syndrome and is present in the environment around SBT leases. Based on our molecular data, there is no evidence of Uronema spp. presence in the infected SBT. This is the first time *M. avidus* has been documented in Australia.

4.2 Introduction

Scuticociliates are free-living marine organisms which feed on suspended bacteria, microalgae or other protozoa. Under certain circumstances Scuticociliates can behave as opportunistic histophagous parasites of marine fish (Elston et al., 1999; Lee et al., 2004; Moustafa et al., 2010b). They are considered to be serious pathogens in fish mariculture (Budiño et al., 2011; Gao et al., 2012; Gao et al., 2010; Whang et al., 2011) and were responsible for causing mortalities in Olive Flounder *Paralichthys*

olivaceus (see Iglesias et al., 2001; Jung et al., 2005; Moustafa et al., 2010b), Turbot *Scophthalmus maximus* (see Dyková and Figueras, 1994; Iglesias et al., 2001; Sterud et al., 2000; Whang et al., 2013), Sea Bass *Dicentrarchus labrax* (see Whang et al., 2013), Southern Bluefin Tuna *Thunnus maccoyii* (SBT) (Munday et al., 1997, 2003), New Zealand Grouper *Polyprion oxygeneios* and Yellowtail Kingfish *Seriola lalandi* (Smith et al., 2009).

The SBT industry is a high value aquaculture sector situated off Port Lincoln, South Australia. The industry practices purse seine fishing to collect 2–4 year age class SBT in the Great Australian Bight, towing the fish back to the commercial lease sites and fattening over a 6 month period. Marine Scuticociliates are the causative agent of swimmer syndrome in SBT, the disease typically occurs between May and November when water temperature drops below18 °C and, in most cases is associated with water temperature below 15 °C (Deveney et al., 2005; Nowak et al., 2007b). Clinical signs include abnormal and vigorous swimming at the surface followed by death (Munday et al., 1997). Affected fish are characterised by significant pathological changes in the olfactory rosettes and brain (encephalitis) along with the presence of Scuticociliates. These Scuticociliates have been identified as *Uronema nigricans* based on morphological analysis of cultures from cerebrospinal fluid (CSF) of infected SBT (Munday et al., 2003).

In 2003, 58% of SBT mortalities examined from a mortality outbreak in winter were identified as being positive for Scuticociliates based on the presence of Scuticociliates in CSF (Deveney et al., 2005). This outbreak was affecting SBT from pontoons belonging to one company. Improved husbandry and feeding practices in the industry have decreased disease prevalence, however sporadic outbreaks still occur (Deveney et al., 2005; Nowak, 2007a; Nowak et al., 2007b).

The use of morphological features to identify Scuticociliates can be difficult (Jung et al., 2007; Song et al., 2009a). Scuticociliates display considerable morphological plasticity, especially when cultured in vitro (Budiño et al., 2011; Salinas et al., 2012). Molecular techniques can be used to identify Scuticociliates (Whang et al., 2013). Analysis of ribosomal RNA (rRNA) subunits has proven useful for identification and comparison of closely related organisms, including congeners (Elwood et al., 1985; Hillis and Dixon, 1991), while the application of mitochondrial barcoding of cytochrome *c* oxidase 1 (cox1) gene has been increasingly used for species delineation (Hebert et al., 2003; Robba et al., 2006; Roe and Sperling, 2007). The use of sequencing data of rRNA genes and cox1 is a universally applicable tool that makes it possible to identify Scuticociliates and confirm taxonomic relationships previously established on the basis of ultrastructural and other morphological characteristics (Budiño et al., 2011; Elwood et al., 1985; Hillis and Dixon, 1991; Jung et al., 2005; Whang et al., 2013).

In this study we document the molecular characterisation of Scuticociliates isolated from CSF of suspected swimmer syndrome SBT mortalities and record the first identification of the Scuticociliate *Miamiensis avidus* (senior synonym of *Philasterides dicentrarchi* (Budiño et al., 2011; Jung et al., 2007)) from SBT (new host) in Australia (new location). We provide data that the Scuticociliates isolated from SBT are distinct from the previously reported agent of swimmer syndrome *Uronema nigricans*.

4.3 Materials and methods

4.3.1 Field collection and processing of Southern Bluefin Tuna

During 2015, 16 wild SBT caught from Pedra Branca off the Southern coast of Tasmania (45°51′00″S 146o58′12″E), were collected by long line fishing. Individual olfactory rosettes were removed from the 16 SBT. In addition, 23 cerebrospinal fluid (CSF) samples (olfactory rosettes not available) were collected from ranched SBT which exhibited signs of swimmer syndrome, such as vigorous swimming inside the pontoon or at intervals swimming up to the surface and sinking again. SBT were opportunistically sampled and ranged between 15 and 40 kg. CSF was sampled from fresh mortalities and moribund individuals using plastic Pasteur pipettes during the 2015 harvesting season from May to the second week of August, off Port Lincoln, South Australia (34°43′56″S 135°51′31″E). Prior to storage at −20 °C, CSF subsamples were examined for the presence of live Scuticociliates. Wet preparations of subsamples were examined and observed using a bright-field microscope at 100× and 400× and most were found to be positive. Samples were considered positive for Scuticociliates if the microscopic observation revealed motile cells that were robust and granular in appearance (Jung et al., 2007) and pyriform shape (morphology consistent for Scuticociliates). All samples (olfactory rosettes and CSF) were stored in nucleic acid preservation solution (4 M ammonium sulphate, 25 mM sodium citrate, 10mMEDTA; pH5.5) in a 1:5 ratio. All samples were stored on ice at approximately 4 °C overnight and then at −20 °C in the laboratory.

4.3.2 Nucleic acid extraction and molecular analysis

Total nucleic acid (TNA)was extracted from the RNA-later preserved olfactory rosettes and CSF samples using Bioline Isolate II Genomic DNA Kit (Bioline, Taunton, MA, USA), following the manufacturer's instructions. TNA quantity of each sample was estimated using spectrophotometry (NanoDrop, Thermo Scientific, Waltham MA,

USA). Additionally, DNA samples, courtesy of Prof. Iva Dyková (Brno University, Czech Republic), from cultured Scuticociliates (Dyková et al., 2010) were provided; two of which were isolated in conjunction with *Neoparamoeba perurans* from fish hosts *Psetta maxima* (CESP/I) and *Salmo salar* (CTAS/I), and two from seaweeds *Lithophyllum racemus* (CLIT/I) and *Palmaria palmata* (CPAL2/1). These samples were also used for the molecular analysis and sequence comparison to Scuticociliates from SBT. Partial fragments of the mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) gene (360 nucleotides) and the small subunit rDNA (SSU rDNA) (507 nucleotides) sequence were amplified using oligonucleotide primers previously reported by Jung et al., 2005 and Whang et al. (2013) (Table 4.1).

PCR was performed in a final volume of 25 µl containing, 10 pM of each primer, 0.5 U of DNA Taq Polymerase (Bioline, Taunton, MA USA), 1.5 mM MgCl2, 0.5 mM dNTPs, stabilizers and enhancers (MyTaq, Bioline, Taunton, MA USA), and DNA template. PCR amplification was performed in a Bio-Rad C1000 Touch Thermal Cycler (Bio- Rad, Hercules, CA USA). The following conditions were used to amplify partial SSU rDNA fragment, using primers Cil 2/Cil 4: at 95 °C for 1 min (activation) and 40 cycles of 95 °C for 15 s (denaturing), 50 °C for 15 s (annealing), 72 °C for 15 s (extension) and a final hold at 4 °C. The PCR conditions used to amplify a partial SSU rDNA fragment using Cil 3/Cil 4 were 95 °C for 1 min (activation) and 35 cycles of 95 °C for 15 s (denaturing), 50 °C for 15 s (annealing), 72 °C for 15 s (extension) and 4 °C as a final hold. The PCR conditions used to amplify a partial sequence of the cox1 gene of M. avidus using primers OX09-142, OX09- 143 and a partial fragment of the cox1 gene of *U. marinum* using OX09-144, OX09-145 were: 95 °C for 1 min (activation) and 30 cycles of 95 °C for 15 s (denaturation), 50 °C for 15 s (annealing) 72 °C for 15 s (extension) and 4 °C as a final hold.

Amplified products were analysed in a 1.5% agarose gel by electrophoresis, stained with ethidium bromide and visualized using the UV transilluminator Gel Doc XR + System (Bio-Rad, Hercules, CAUSA). Amplified products were purified using the ISOLATE II PCR and Gel Purification kit (Bioline, Taunton, MAUSA) and concentration was measured by spectrophotometry (NanoDrop, Thermo Scientific, Waltham MA, USA). Both strands, forward and reverse of the PCR products, were sequenced using ABI Big Dye Terminator v3.1 chemistry (Applied Biosystems, Foster City, CA USA) by the Australian Genome Reference Facility (AGRF), Parkville, Victoria.

Table 4.1. Oligonucleotides used in polymerase chain reactions for this study this study.

Primer	Sequence	Target	Source
OX09-142	5'- AGTAATAATAGAACATTTAACGAATTTAATAACAC	*M. avidus cox1*	Whang *et al.* 2013
OX09-143	5'- CGTCTTGTAATTAATAAATTTGTAAACGATAC AACATAGAGCATATAGAGAGTACTCTAA	*M. avidus cox1*	
OX09-144	5'- AACATAGAGCATATAGAGAGTACTCTAA	*U. marinum cox1*	
OX09-145	5'- TTCATCCAGCTGTTGTTAATGT	*U. marinum cox1*	
Cil 2	5'- CTATCAGCTTTCGATGGT	SSU rRNA of Scuticociliates	Jung *et al.* 2005
Cil 3	5'- GTAGGCTCTTTACCTTGA	SSU rRNA of Scuticociliates	
Cil 4	5'- CAAATCACTCCACCAACT	SSU rRNA of Scuticociliates	

4.3.3 Alignment and phylogenetic analysis

Sequencher™ (GeneCodes Corp., Ann Arbor, Michigan, U.S.A., ver. 5.2.4) was used to produce consensus sequences from corresponding forward and reverse complemented sequences. Scuticociliate sequences from the appropriate gene regions (partial SSU rDNA and mt *cox 1*) were obtained via BLAST search (Altschul

et al., 1990) (Table 4.2 and 4.3) and aligned in conjunction with the sequences obtained as part of this study using the ClustalX (Thompson et al., 1997) accessory application in Bioedit® (Hall, 1999). Alignments were further refined by eye. Sequences produced as part of this study are listed by locality (Port Lincoln) and numbered based on their sample number. Bayesian Inference analyses of sequence alignments were conducted using; MrBayes® ver. 3.2.2 (Ronquist and Huelsenbeck, 2003) using the parameters: ngen = 2.000,000, nst = six, four Markov Chains used, burn-in was set to 100 and every 100th tree saved. The trees were based on a 50% majority rule consensus as per Aiken et al. (2007). The trees produced from Bayesian analyses were viewed using Figtree® (http://tree.bio.ed.ac. uk/software/figtree/). Multiple pairwise alignments were produced in Mega version 6 (Tamura et al., 2013) using number of differences. Partial SSU rDNA and mt Cox 1 sequences generated from this study were deposited in Genbank under the accession numbers KX842459-KX842468 (*M. avidus* SSU rDNA), KX842469-KX842477 (*M. avidus* mt Cox 1) and KX842478-KX842483 (*U. marinum* mt Cox 1).

Table 4.2. Short sub-unit ribosomal DNA (SSU rDNA) Scuticociliate sequences used in this study (not including those collected from SBT the present study).

Taxonomic name	Name in phylogenetic tree	GenBank accession no.
Anophyroides haemophila	*Anophyroides haemophila*	U51554.1
Cohnilembus verminus	*Cohnilembus verminus* isolate FXP	HM236339.1
Entorhipidium tenue	*Entorhipidium tenue*	AY541688.1
Homalogastra setosa	*Homalogastra setosa* isolate GT1	EF158848.1
Mesanophrys carcini	*Mesanophrys carcini*	AY103189.1
Metanophrys similis	*Metanophrys similis*	AY314803.1
Me. sinensis	*Metanophrys sinensis* isolate FXP	HM236336.1
Miamiensis avidus	*Miamiensis avidus* 1	AY550080.1
	Miamiensis avidus 2	AY642280.1
	Miamiensis avidus isolate JM1	JN689229.1
	Miamiensis avidus isolate JM2	JN689230.1
	Miamiensis avidus strain A3	EU831193.1
	Miamiensis avidus strain GJ01	EU831199.1
	Miamiensis avidus strain JJ3	EU831194.1
	Miamiensis avidus strain JJ4	EU831198.1
	Miamiensis avidus strain SJF-03B	EU831195.1
	Miamiensis avidus strain SJF-06A	EU831196.1
	Miamiensis avidus strain WD4	EU831192.1
	Miamiensis avidus strain *I*1	JX914665.1
	*Miamiensis avidus** strain SNUSS001	GU572375.1
Miamiensis sp.	*Miamiensis* sp. 2 PJS-2009	FJ936000.1
Paranophrys magna	*Paranophrys magna*	AY103191.1
Parauronema longum	*Parauronema longum*	AY212807.1
Philaster apodigitiformis	*Philaster apodigitiformis*	FJ648350.1
Philasterides armatalis	*Philasterides armatalis* strain G	FJ848877.1
Plagiopyliella pacifica	*Plagiopyliella pacifica*	AY541685.1
Porpostoma notata	*Porpostoma notata* isolate FXP	HM236335.1
Pseudocohnilembus hargisi	*Pseudocohnilembus hargisi*	AY833087.1
P. longisetus	*Pseudocohnilembus longisetus*	FJ899594.1
P. persalinus	*Pseudocohnilembus persalinus* 3	AY835669.1
	Pseudocohnilembus persalinus 1	AY551906.1
	Pseudocohnilembus persalinus 2	GU584096.1
	Pseudocohnilembus persalinus isolate wyg	GQ265955.1
Schizocaryum dogieli	*Schizocaryum dogieli*	AF527756.1
Tetrahymena pyriformis	*Tetrahymena pyriformis* (Outgroup)	X56171.1
Thyrophylax vorax	*Thyrophylax vorax*	AY541686.1
Uronema marinum	*Uronema marinum* 2	Z22881.1
	Uronema marinum 1	AY551905.1
	Uronema marinum strain CESP/1	GQ259744.1
	Uronema marinum strain CLIT/I	GQ259745.1
	Uronema marinum strain CPAL2/I	GQ259746.1
	Uronema marinum strain CTAS/I	GQ259747.1
	Uronema marinum strain JK1	DQ867072.1
	Uronema marinum strain JK2	DQ867073.1
	Uronema marinum strain JK3	DQ867074.1
Uronema sp.	*Uronema* sp. 1 PJS-2009	FJ936001.1
Uronema sp.	*Uronema* sp. WS-2012 isolate XY2009113003	JN885088.1
U. filificum	*Uronemella filificum*	EF486866.1

* submitted to Genbank as *Philasterides dicentrachi* (a junior synoynm of *M. avidus*).

Table 4.3. Mitochondrial cytochrome *c* oxidase 1 (mt *cox1*) Scuticociliate sequence used in analysis (not including those from present study).

Taxonomic name	Name in phylogenetic tree	GenBank accession no.
Entodiscus borealis	*Entodiscus borealis* isolate OLI	FJ905123.1
Miamiensis avidus	*Miamiensis avidus* 2	GQ855300.1
	Miamiensis avidus isolate IUET-AK26P	KP170494.1
	Miamiensis avidus strain A3	EU831214.1
	Miamiensis avidus strain Iyo-1	EU831227.1
	Miamiensis avidus strain Mie0301	EU831233.1
	Miamiensis avidus strain Nakajima	EU831226.1
	Miamiensis avidus strain SJF-03B	EU831216.1
	Miamiensis avidus strain WD4	EU831213.1
	Miamiensis avidus strain YS3	EU831218.1
	Miamiensis avidus 1	GQ342957.1
Pseudocohnilembus longisetus	*Pseudocohnilembus longisetus*	GQ500580.1
P. persalinus	*Pseudocohnilembus persalinus* 1	GQ500579.1
	Pseudocohnilembus persalinus 2	GU584095.1
Tetrahymena pyriformis	*Tetrahymena pyriformis* strain E (Outgroup)	EF070300.1
Uronema marinum	*Uronema marinum*	GQ500578.1

4.4 Results

4.4.1 Molecular identification of Scuticociliates

Nineteen samples from CSF of ranched SBT were identified as positive for Scuticociliates using PCR. None the olfactory rosettes samples from wild SBT showed amplification for either SSU rDNA or mt *cox1*. These samples were considered negative. The Scuticociliates isolated from CSF of SBT were 100% identical based on both partial SSU rDNA and mt *cox1*. Both partial SSU rDNA and mt *cox1* showed that the Scuticociliates isolated from SBT CSF were identical to published sequences of *M. avidus* and differed from *Uronema* spp. sequenced as part of this study and previously published sequences; partial SSU rDNA differed by 23–24 nucleotides and 95% similarity, and mt *cox1* differs by a range of 34–35 nucleotides and is 87.5–90%

in range of similarity. Our sequencing results for SSU rDNA of *U. marinum* isolates from Dyková et al. (2010) showed that their sequences were 100% identical to those generated by Dyková et al. (2010) from the same samples. All *U. marinum* SSU rDNA sequences obtained in this study were 100% identical.

4.4.2 Bayesian Inference analyses

Bayesian Inference analyses of partial SSU rDNA (Figure 4.1) showed that the ten sequences from SBT ranched at Port Lincoln were 100% identical and homologous to published SSU rDNA sequences of *M. avidus* (Jung et al., 2011b; Jung et al., 2007; Paramá et al., 2006; Salinas et al., 2012). The SBT samples and *M. avidus* formed a clade with all other taxa (to the exclusion of the *Uronema* clade including those sequenced as part of this study (samples from Dyková et al., 2010)).

The partial mt *cox1* Bayesian Inference analysis (Figure 4.2) showed a similar topology to the SSU tree in that *M. avidus* formed a clade with *Pseudocohnilembus* spp. to the exclusion of the *Uronema* + *Entodiscus borealis* clade. Within the *M. avidus* + *Pseudocohnilembus* spp. clade, *M. avidus* formed a clade to the exclusion of *Pseudocohnilembus* spp.; the samples collected from Pt Lincoln as part of this study formed a clade to the exclusion of all other *M. avidus*.

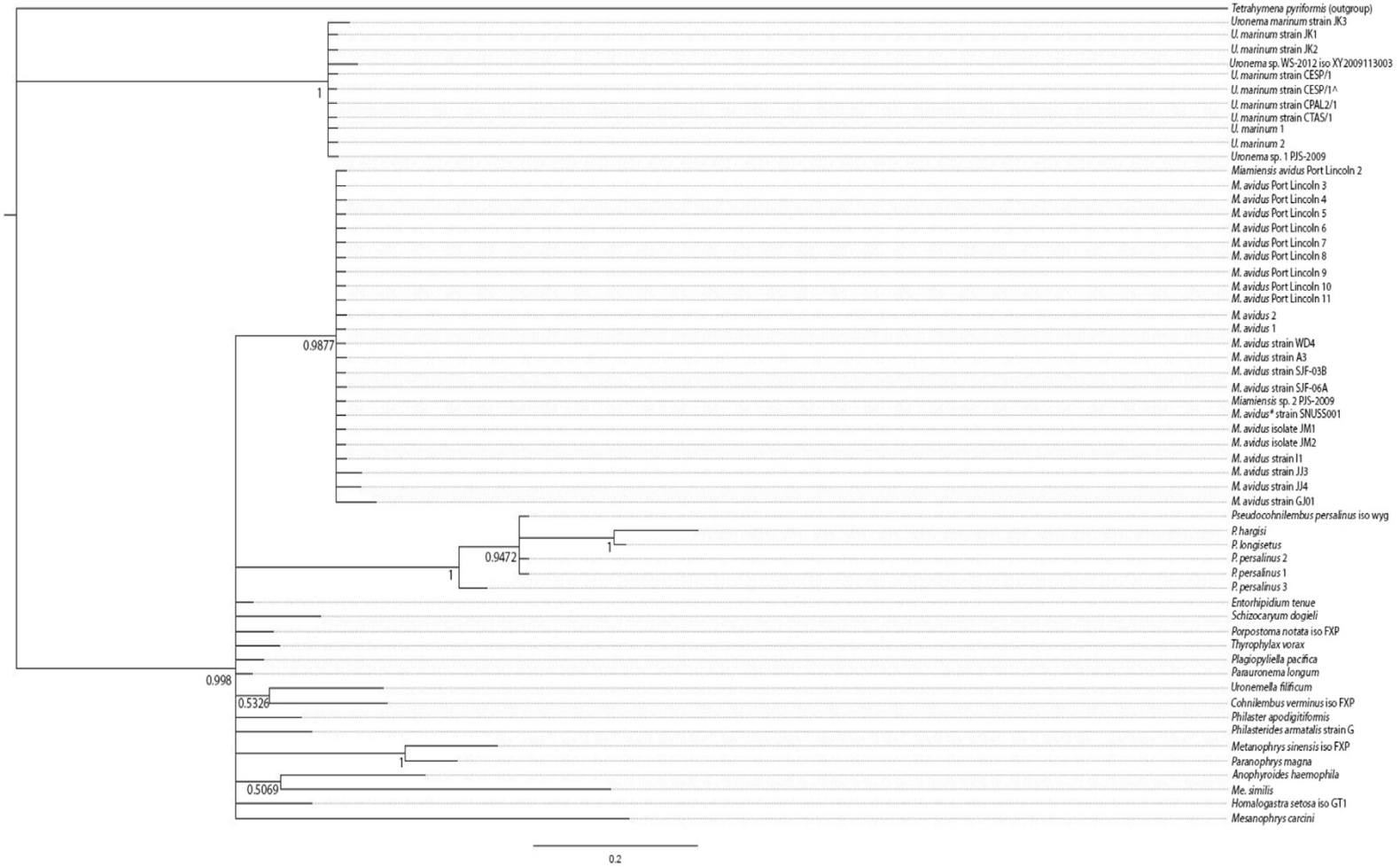

Figure 4.1. Bayesian Inference analysis of partial SSU rDNA of Scuticociliates via MrBayes v 3.2.2. *Tetrahymena pyriiformis* was the outgroup.

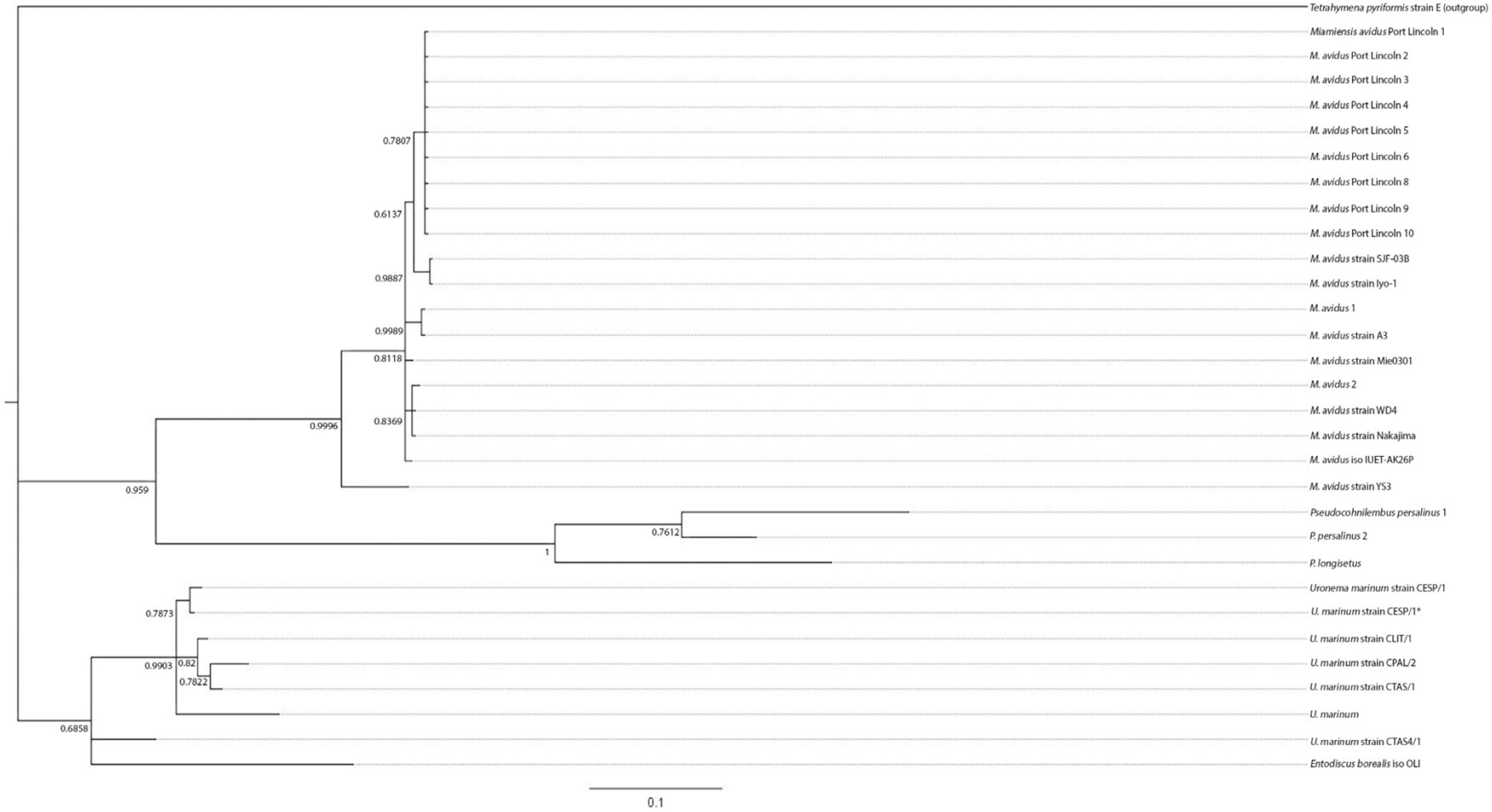

Figure 4.2. Bayesian Inference analysis of partial mitochondrial cytochrome *c* oxidase 1 gene of Scuticociliates via MrBayes v 3.2.2. *Tetrahymena pyriiformis* was the outgroup.

4.5 Discussion

The comparison of SSU rDNA and mt *cox1* sequences of Scuticociliates collected from SBT and *U. marinum* in association with comparison to published Scuticociliate sequences showed that in the swimmer syndrome cases we studied, *M. avidus* was present and *U. nigricans* which was identified during earlier SBT ranching seasons as a cause of swimmer syndrome (Crosbie and Munday, 1997; Deveney et al., 2005; Munday et al., 1997, 2003), was absent. Previous identification was based on morphology of cultures of the Scuticociliates from SBT CSF (Munday et al., 1997) and it has been shown that morphology of Scuticociliates can change in culture (Fenchel, 1990; Jee et al., 2001). Our phylogenetic analyses of 2 gene regions (SSU rDNA and mt *cox1*) (Figure 4.1 and 4.2) that are subjected to different evolutionary pressures (Gao et al., 2012; Jung et al., 2011b) clearly showed that the samples isolated from SBT swimmer syndrome samples were *M. avidus*, and based on these analyses they were not particularly close in sequence similarity to the *Uronema* sp. sequenced in this study.

Our Bayesian Inference analyses support the work of Gao et al. (2012) in illustrating that the genera belonging to Philasterida: *Miamiensis* and *Uronema* are consistently positioned within different clades. Our study differed from Gao et al. (2012) in that we have analysed only within the Philasterida while they compared SSU rDNA of a wide range of orders within the Scuticociliata. The partial SSU rDNA analysis positioned the *Uronema* clade to the exclusion of all other Scuticociliates as opposed to that of the complete SSU rDNA in Gao et al. (2012); this may be an artefact of not analysing the complete gene region.

While our results show that M. avidus is associated with swimmer syndrome in SBT further investigation is required to determine if *Uronema nigricans* (or indeed

other opportunistic Scuticociliate species) is also associated with swimmer syndrome in ranched SBT. If multiple species are causative agents and *U. nigricans* is also associated with swimmer syndrome in SBT (as described by Munday et al., 1997) then an investigation is warranted into whether environmental cues favour infection by one Scuticociliate species over another. It is plausible that both Scuticociliate species (*M. avidus* and *U. nigricans*) are responsible as they are opportunistic parasites that may both be present in the SBT ranching environment. Mixed isolates of Scuticociliates, including *M. avidus*, *U. marinum* and *Parauronema virginianum* were obtained from infected New Zealand grouper, *Polyprion oxygeneios* (see Salinas et al., 2011). Smith et al. (2009) isolated from dead *P. oxygeneios* and identified using SSU rRNA both *M. avidus* and *U. marinum*. However, in some host species only *M. avidus* has been suggested as the cause of Scuticociliatosis. Only *M. avidus* induced histological changes when olive flounder *Paralichthys olivaceus* were experimentally infected with *M. avidus*, *Pseudocohnilembus persalinus*, *P. hargisi* and *U. marinum* (Song et al., 2009a). This suggested that *P. persalinus*, *P. hargisi* and *U. marinum* were not causative agents of Scuticociliatosis in olive flounder, although all three species have been reported and identified in the affected fish (Song et al., 2009a) and Smith et al. (2009) reported that *U. marinum* caused mortalities in *Seriola lalandi* in New Zealand. Culture conditions may promote growth of one species, and in some cases that species may not be the pathogen. For example, cultures of amoebae from the gills of Amoebic Gill Disease-affected Atlantic salmon resulted in isolation of *N. pemaquidensis* and *N. branchiphila*, while it was later shown that *N. perurans* is the only causative agent (Crosbie et al., 2012; Young et al., 2007). Further investigation is required to assess the broad pathogenicity of a range of Scuticociliates believed to cause Scuticociliatosis in finfish. This study is the first to document the presence of *M.*

avidus in Australia. It is yet to be established how wide spread *M. avidus* is in Australia's marine environment.

4.6 Acknowledgements

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Chapter five

Detection of *Miamiensis* avidus (Ciliophora: Scuticociliata) and *Cardicola* spp. (Trematoda: Aporocotylidae) DNA in biofouling from Southern Bluefin Tuna, *Thunnus maccoyii* pontoons off Port Lincoln, South Australia

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Published in:

Power, C., Balli-Garza, J., Evans, D., Nowak, B.F., Bridle, A.R., Bott, N.J., 2018. Detection of *Miamiensis avidus* (Ciliophora: Scuticociliatia) and *Cardicola* spp. (Trematoda: Aporocotylidae) DNA in biofouling from Southern Bluefin Tuna, pontoons off Port Lincoln, South Australia. Aquaculture 502, 128–133.

5.1 Abstract

Presence of biofouling on pontoons and other structures can have adverse effects on fish health, both by affecting water quality and acting as a reservoir for pathogens. This study focused on three species of parasites affecting Southern Bluefin Tuna (*Thunnus maccoyii*) (SBT): the blood flukes (*Cardicola forsteri* and *Cardicola orientalis*) and the Scuticociliate *Miamiensis avidus.* Blood flukes are the main health concern for SBT. They have two free living stages (miracidium and cercaria) and their intermediate host can be present in biofouling. *Miamiensis avidus* (Ciliophora: Scuticociliata) is an opportunistic pathogen thought to be the causative agent of swimmer syndrome in SBT. To determine if biofouling is a reservoir for blood flukes or *M. avidus* six perspex plates and six net pieces were deployed in two SBT pontoons at one and four meter depths and sampled one and three months after deployment. Biofouling samples were rarely positive for blood fluke DNA based on qPCR detection, but they were most frequently detected on plate samples at 3 months. Prevalence of *Miamiensis avidus* based on qPCR detection increased from 38% at one month to 89% at three months. No significant difference was observed between depths at which the plates were deployed. *M. avidus* was detected from total DNA extracted from a wide range of taxonomic groups collected from the fouling samples. Results suggest that biofouling may act as a reservoir for *M. avidus* in aquaculture. Monitoring environmental reservoirs may be an important non-destructive surveillance tool. Further optimization of the detection in biofouling may provide insights into host-pathogen interactions which will inform aquatic animal health management. This approach could also be applied to the surveillance of other potential aquaculture pathogens affecting species farmed in marine pontoons.

5.2 Introduction

Biofouling is composed of naturally forming marine communities that inhabit artificial structures in marine environments. Biofouling is associated with aquaculture infrastructure, and forms on sea pontoons. A range of fish pathogens have been associated with biofouling, for example, Scuticociliates have been previously identified in biofouling communities (Shimeta et al. 2012). The intermediate host infected by blood flukes was found in biofouling on tuna farms in Japan (Sugihara et al. 2014; Shirakashi et al. 2016; Ogawa et al. 2017). Presence of parasites in biofouling could support the role of biofouling as a reservoir for parasites and determine the risk of parasitic infections. Biofouling organisms can also function as a potential reservoir for parasites and other diseases that can affect fish (Fitridge et al. 2012; Swain and Shinjo 2014; Tan et al. 2002). Several parasitic diseases affecting farmed fish have been linked to the presence of biofouling associated with sea pontoons such as amoebic gill disease (AGD) in Atlantic Salmon (Clark and Nowak 1999; Tan et al. 2002) and the monogenean *Heterorobothrium okamotoi* which affects Tiger Puffer (Ogawa and Inouye 1997; Ogawa et al. 2005). Additionally, viral and bacterial agents that cause illness in finfish have been found in the tissue of biofouling organisms, these include 13p2 reovirus, JOV-1 Japanese oyster virus, chum salmon virus, infectious hematopoietic necrosis virus and bacteria like *Vibrio* spp. which are commonly found in bivalves (Meyers 1984; Fitridge et al. 2012).

Blood flukes can cause significant impacts in the finfish aquaculture industry, especially in ranched tuna (Colquitt et al. 2001). *Cardicola forsteri* and *C. orientalis* are the two species of blood flukes that have been identified in Southern bluefin tuna (SBT). The adults of *C. forsteri* are present in the heart, where the eggs are released, travelling through the circulatory system and eventually reaching the gills. Adults and

eggs of *C. orientalis* can be found in the gills, accumulating in the afferent arteries (Palacios-Abella et al. 2015; Polinski et al. 2014; Shirakashi et al. 2012). The presence of *C. forsteri* eggs in the gills have been shown to cause severe branchitis, respiratory distress, increased branchial mucus and lethargy, leading to mortalities (Cribb et al. 2011; Dennis et al. 2011). *Cardicola orientalis* eggs can clog the filament arteries in the gills, obstructing the normal blood flow (Polinski et al. 2013; Shirakashi et al. 2013).

Fish blood flukes have an indirect life cycle, reproducing in an invertebrate intermediate host and in a definitive fish host. In Australia, the terebellid polychaete *Longicarpus modestus* has been identified as the intermediate host for *C. forsteri* (Cribb et al. 2011). Recent studies found that another terebellid, *Neomphitrite vigintipes*, is an intermediate host for *C. forsteri* in Japan, and even though fish blood flukes are highly host specific, it is well known that some have more than one intermediate host allowing them to expand their geographical distribution (Ogawa et al. 2011; Shirakashi et al. 2016; Ogawa et al. 2017). In the case of *C. orientalis, Nicolea gracilibranchis* has been described as its intermediate host in Japan (Shirakashi et al., 2016). Polychaetes are usually part of biofouling communities, many of them having a worldwide distribution (Fitridge et al. 2012). They are often attached to nets, ropes and floats on fish pontoons (Fitridge et al. 2012; Shirakashi et al. 2016). Presence of parasites such as blood flukes are higher in ranched SBT than in wild SBT, suggesting that conditions for completing their life cycle are present in, or in close proximity to the aquaculture environment (Cribb et al. 2011; Palacios-Abella et al. 2015).

Scuticociliates are free-living marine organisms which feed on suspended bacteria, microalgae or other protozoa. Under certain circumstances some Scuticociliates can behave as opportunistic histophagous parasites of marine

organisms (Harikrishnan et al. 2010). Scuticociliates can be pathogenic in fish mariculture and have been responsible for causing mass mortalities in Olive Flounder *Paralichthys olivaceus* (Jung et al. 2007), Turbot *Scophthalmus maximus* (Dyková and Figueras 1994; Iglesias et al. 2001), Sea Bass *Dicentrarchus labrax* (Dragesco et al. 1995), Southern Bluefin Tuna *Thunnus maccoyii* (Munday et al. 1997, 2003), New Zealand Grouper *Polyprion oxygeneios* and Yellowtail Kingfish *Seriola lalandi* (Smith et al. 2009). *Miamiensis avidus* is considered one of the most common Scuticociliate pathogens of finfish (Song et al. 2009a ; Whang et al. 2013). It has been reported from the cerebrospinal fluid (CSF) of infected SBT in pontoons off Port Lincoln, South Australia (Balli Garza et al. 2017).

Marine Scuticociliates are the causative agent of swimmer syndrome in SBT (Munday et al. 1997; Deveney et al. 2005; Nowak et al. 2007b). Swimmer syndrome typically occurs between May and November when water temperature drops below 18°C and, in most cases is associated with water temperature below 15°C (Deveney et al. 2005). Clinical signs include abnormal and vigorous swimming at the surface followed by death (Munday et al. 1997). Affected fish are characterised by significant pathological changes in the olfactory rosettes and brain (encephalitis) along with the presence of Scuticociliates. In 2003, 58% of SBT mortalities examined from a mortality outbreak in winter were identified as being positive for Scuticociliates based on the presence of Scuticociliates in CSF (Deveney et al. 2005). This outbreak was affecting SBT only from one company. Improved husbandry and feeding practices in the industry have decreased disease prevalence, however sporadic localised outbreaks of swimmer syndrome still occur (Deveney et al. 2005; Nowak 2007a; Nowak et al. 2007b).

The SBT industry is an aquaculture sector, which practices purse seine fishing to collect 2 - 3 year age class SBT in the Great Australian Bight, towing fish back to growout sites and fattening over a 6 month period (Balli Garza et al. 2017). Both blood flukes (*Cardicola forsteri* and *C. orientalis*) and to a lesser extent swimmer syndrome have been causing health issues in ranched SBT. In this study we investigated the effect of time (age of biofouling and SBT time in ranching), depth and pontoon on the presence of DNA of blood flukes *Cardicola forsteri and C. orientalis* and the Scuticociliate, *M. avidus* in biofouling.

5.3 Materials and methods

5.3.1 Study site and sample collection

Mesh panels were set up on two SBT sea pontoons in Port Lincoln, South Australia (34°73'02" S 135°85'05" E), approximately 6km offshore of mainland, nets were 250 m apart, at right angles to current flow. SBT were transferred to pontoons in late January 2016 (pontoon A 22nd January, pontoons B 27 January). SBT mortalities were monitored daily as an indicator of SBT performance.

The panels were located at depths of 1 m and 4 m. Each mesh panel contained six Perspex® settlement plates (20cm x 20cm) and six net pieces (4 meshes by 4 meshes square; mesh size 40mm x 40mm). Initial set up was conducted in April 2016, where plates and nets were deployed by SCUBA. After one month, three plates and three net pieces were removed from each pontoon. Samples were immediately put on ice and processed in the laboratory 2 - 4hr after collection. Biofouling was scraped from plates using a sterile knife into a Whirl-Pak® sterilised bag (Sigma-Aldrich, ST Louis, MO, USA), followed by wiping clean with a 2 x 4 cm sterilised Whirl-Pak® Speci-Sponge®, before being returned to the sterilised bag as per Pochon et al. (2015). Net

pieces were stored in sterile polyethylene bags and all samples were stored at -20°C. Remaining plates and net pieces were collected after three months and processing was repeated. Each side of plate samples were considered a separate sample. Overall, 24 net samples and 48 plate samples were collected.

5.3.2 Sample preparation and sorting

Each plate sample was defrosted for up to two hours to allow for taxonomic sorting of invertebrates. Using gloves and a sterile spatula, biofouling was removed from sterilised bags and placed in a sterilised Petri dish. Samples were identified based on morpho-groupings into broad taxonomic levels using an M60 stereomicroscope (Leica, Germany). A time limit of two hours was placed on taxonomic sorting to minimise DNA degradation, and subsequently the entire sample could not be processed. The unidentified material was treated as a mixed biofouling sample. After sorting, taxonomic groups were placed in separate sterile tubes and the mixed biofouling placed in its own tube and stored at -20ºC. Samples were lyophilised (freeze dried) for a period of 24-48 h using the Alpha 1-2 LDplus Freeze Dryer (CHRIST, Germany). After freeze drying, samples were ground to a fine powder with a mortar and pestle and stored at 4ºC until further analysis. Samples were weighed both prior and post freeze drying to the nearest 0.1 g using the XS105 Analytical Balance (Mettler-Toledo, Port Melbourne, VIC).

5.3.3 DNA extraction

Sponges were thawed and 20 ml of UltraPure™ DNase/RNase-free water (Thermo Fisher Scientific, Scoresby, VIC) added, followed by maceration for 2 min using the BagMixer® 400P (Interscience, France). Excess liquid was extracted from

the sponges in a consistent way, applying comparable pressure to all samples. The liquid was centrifuged at 4000 x g for 15 min and the supernatant discarded. The pellet was added to the freeze dried material and DNA was extracted using the PowerMax® Soil DNA Isolation Kit (MO BIO, Carlsbad, CA, USA) following the manufacturer's instructions. Subsamples taken from taxonomic groups were approximately 5 g smaller so the PowerSoil® DNA Isolation Kit (MO BIO, Carlsbad, CA, USA) was used following the manufacturer's instructions. The quantity and quality of samples were estimated using spectrophotometry (Nanodrop™, Thermo Fisher Scientific, Scoresby, VIC).

Using the NanoDrop™ Lite Spectrophotometer, concentration (ng/µl) was measured to determine DNA quantity. All samples had concentration levels sufficient for PCR, with one month samples ranging from 2.3 to 42.1 ng/µl and three month samples ranging from 6.6 to 63.8 ng/µl. Spectrophotometry was also used to determine A_{260}:A_{280} ratios as a measure of DNA purity. All samples had acceptable A_{260}:A_{280} ratios.

5.3.4 Primer and probe design

5.3.4.1 Cardicola forsteri and Cardicola orientalis

The species specific primers and probes used to detect *C. forsteri* and *C. orientalis* were designed in a previous study, which confirmed their specificity (Pennacchi et al. 2016; Neumann et al., 2018). These were targeted against heterogeneous areas of the internal spacer-2 (ITS2) region of rDNA specific to each species available on GenBank (Table 5.1).

5.3.4.2 Miamiensis avidus

The species specific primers and TaqMan® (Roche Molecular Systems Inc.) Minor Groove Binding (MGB) probe (Applied Biosystems, USA) used to detect *M. avidus* were designed using Primer Express® v2.0 (Applera Corporation, USA). These were targeted against the long subunit (LSU) of rDNA (Table 5.1). The primers and probe developed in Primer Express® v2.0 were checked against other related sequences and subjected to a Basic Local Alignment Search Tool (BLAST)(https://blast.ncbi.nlm.nih.gov) to check there were no matches to closely related taxa or to organisms present in the SBT aquaculture environment. The specificity of the qPCR assay has previously been tested with Scuticociliates from the cultured Neoparamoeba from Dykova et al. (2010), and a range of pathogens and invertebrates commonly found in SBT aquaculture and southern Australian waters (the aporocotylid blood fluke *C. forsteri*, gill fluke *H. thynni*, capsalid skin fluke *Benedenia seriolae*, *Caligus* spp., as well as SBT DNA) (Balli Garza et al. 2017).

Table 5.1. Oligonucleotide primers and probes used to amplify *Cardicola spp.* and *M. avidus* for qPCR detection.

Target	GenBank accession #	Name	Amplicon size	Sequence (5'-3')
C. forsteri (ITS-2 rDNA)	EF661575	Cfor_F Cfor_R Cfor_probe	287 bp	TGATTGCTTGCTTTTTCTCGAT TATCAAAACATCAATCGACATC HEX-CCACGACCTGAGCACAAGCCG-BHQ1
C. orientalis (ITS-2 rDNA)	HQ324226	Cori_F Cori_R Cori_probe	191 bp	TGCTTGCTATTCCTAGATGTTTAC AACAACTATACTAAGCCACAA HEX-CACAAGCCGCTACCACAATTCCACTC-BHQ1
M. avidus (LSU rDNA)		Mavi_F Mavi_R Mavi_probe	144 bp	GAAAGCCGTAGAAGAGTAATCAATGA T CAACTTCATTTTGTCTGTGATATTCGA FAM-ACGATGTAAGCGTGTGTC-MGBNFQ

5.3.5 Quantitative PCR

5.3.5.1 Cardicola forsteri and *Cardicola orientalis*

Quantitative PCR assays were performed on a CFX Connect Real-Time PCR Detection System (Biorad, NSW, Australia) in a final volume of 10 µL containing 5 µL of 2xMyTaq™ mix (Bioline), 400 nM of each primer, 150 nM probe, and 2 µl of template DNA. Probes were labelled at the 5′ end with 6-carboxy-2,4,4,5,7,7-hexachlorofluorescein succini-midyl ester (HEX) together with a Black Hole Quencher® (BHQ1, Biosearch Technologies, CA, USA) added to the 3′ terminus. Cycling conditions were 95ºC for 3 min, followed by 45 cycles of 95ºC for 10 s (denaturing) and 60ºC for 30 s (annealing), with relative fluorescence measured at the end of the extension step at 60ºC. All samples were analysed in duplicates, including positive controls and no template control for each run. DNA concentration was calculated for each PCR reaction using the mechanistic model "cm3" (Carr et al. 2012) and the qPCR package within R software v3.2.2 (Ritz and Spiess 2008).

5.3.5.2 Miamiensis avidus

Quantitative PCR assays were performed on a Rotor-Gene™ Q (Qiagen, Hilden, Germany) in a final volume of 20µL containing 10µL of SensiFAST™ Probe 2x No-ROX Mix (Bioline, London, UK), 0.8µL of each 10µM primer (GeneWorks, Thebarton, SA), 2µL of 1µM probe (Thermo Fisher Scientific, Scoresby, VIC), 4.4µL of RNase/DNase free water and 2µL of template DNA. Reaction cycling conditions were: 95ºC for 5 min, followed by 40 cycles of 95ºC for 10 s (denaturing) and 60ºC for 30 s (annealing), with relative fluorescence measured on the annealing cycle. All samples tested were analysed in duplicates, including a positive control and no template control for each run.

For each assay, the limit of detection (LOD) and the limit of quantification (LOQ) were calculated using triplicates of four serial dilutions from a synthetic double stranded gBlock® standard (Integrated DNA Technologies, Iowa, USA), and then converted to copy number per mass using a nucleotide copy number calculator (http://www.endmemo.com/bio/dnacopynum.php).

To detect effects of time, depth and pontoon on the presence of *M. avidus*, three-way analysis of variance (ANOVA) used to analyse interaction between factors. Results were considered statistically significant when $p \leq 0.05$. Assumptions of ANOVA were tested using the Shapiro-Wilk test for normality. Statistical analysis was performed using Minitab16™.

5.4 Results

Within 24 net and 48 plate mixed biofouling samples, eight broad taxonomic groups were identified – Bivalvia, Bryozoa, Gastropoda, Polychaeta, Amphipoda, Ascidiacea, Cirripedia and Anthozoa. The main group identified during taxonomic

sorting were bivalves with 39 (81.25%) plate sub samples and 18 (75%) net sub samples. From the rest of the groups, twenty amphipod samples were collected, six bryozoan, five polychaete, three ascidian, and single samples of barnacle (Cirripedia), sea anemone (Anthozoa), and gastropod were identified. Compositions seen on plate and net samples was similar with dominance of bivalves and amphipods, as well as large amounts of red and green algae and unidentifiable zooids. No bryozoans were found on plate substrates and no barnacles, sea anemones, tunicates or gastropods were found on net substrates.

Biofouling samples were rarely positive for blood fluke DNA, with one positive sample at one month and seven positive samples at three months out of 166 samples tested (Table 5.2 and 5.3). Only 2.4% of samples were positive for *C. forsteri* and 2.4% were positive for *C. orientalis*. The highest copy number for *Cardicola* spp. was detected in mixed biofouling for *C. forsteri* reaching 14,230,830.8 copies/mg (Table 5.2). Individual samples of amphipods, bivalves and ascidians were positive for *C. forsteri*. Three mixed biofouling samples were positive for *C. orientalis* DNA at three months with the highest 1,790,741.7 copies/mg. *Cardicola orientalis* was also detected in an individual amphipod sample at one month. Two samples positive for *C. orientalis* were from net biofouling, whilst all remaining positive samples were from plates. All *C. forsteri* DNA positive samples were from 4 m depth, whereas only one *C. orientalis* positive sample was from 4 m, the remaining three samples were from 1 m (Table 5.3). Most positive samples were from pontoon A, whilst only 2 samples were positive for blood fluke DNA in pontoon B, and both at three months (Table 5.3). While the cumulative SBT mortalities during whole ranching period were similar in pontoon A (2.2%) and pontoon B (2.5%), 51% of all mortalities occurred in May in pontoon A whereas pontoon B mortalities in May were only 21% of total with 38% of total

mortalities occurring in July. This suggests that there were differences in health status of SBT between the two pontoons.

Table 5.2. Samples analysed for the presence of *C. forsteri* and *C. orientalis* DNA and the number of positive samples.

Sample	n	Total positive sample	Result	Number of positive sample
Mixed biofouling	72	4	*C. forsteri*	1
			C. orientalis	3
Bivalve	57	1	*C. forsteri*	1
Amphipod	20	2	*C. forsteri*	1
			C. orientalis	1
Ascidian	2	1	*C. forsteri*	1
Bryozoa	7	0	Negative	0
Polychaete	5	0	Negative	0
Gastropod	1	0	Negative	0
Anthozoa	1	0	Negative	0
Barnacle	1	0	Negative	0
Tunicate	1	0	Negative	0

Table 5.3. Effect of sampling time, depth and substrate on the presence of *C. forsteri* and *C. orientalis* in biofouling. In each case one sample was positive. + ve – samples positive for blood fluke DNA but too low to quantify.

Time (month)	Depth (m)	Substrate	Sample	Pontoon	Species	DNA copy number / mg ww
1	4	Plate	Amphipod	A	*C. forsteri*	46
3	4	Plate	Mixed biofouling	A	*C. forsteri*	14,230,831
3	4	Plate	Bivalve	A	*C. forsteri*	4
3	4	Plate	Ascidian	B	*C. forsteri*	2
3	1	Plate	Mixed biofouling	A	*C. orientalis*	1,790,742
3	4	Plate	Mixed biofouling	A	*C. orientalis*	+ve
3	1	Net	Mixed biofouling	B	*C. orientalis*	97,376
3	1	Net	Amphipod	A	*C. orientalis*	+ve

Prevalence of *M. avidus* in mixed biofouling plate samples increased from 17% at one month to 83% at three months, and in mixed biofouling net samples from 83% at one month to 100% in three months (Table 5.4). *Miamiensis avidus* DNA copy numbers decreased over time, with ranges of 650.0 - 5174.0 copies/mg at one month and 337.3 - 5042.1 copies/mg at three months. *M. avidus* was detected in all other morpho-groupings collected from biofouling samples (Table 5.5). When comparing overall prevalence of *M. avidus*, there was an increase from 38% at one month to 89% at three months. However, when comparing samples at different depths or pontoons, no change in prevalence was seen. Time had a significant effect on the prevalence of *M. avidus* ($p \leq 0.000$), but depth ($p=1.000$) or pontoon ($p=0.573$) had no effect. No statistically significant three-way interaction between time, depth and pontoon was seen ($p=0.519$).

Table 5.4. Prevalence (%) and copy numbers of *M. avidus* at Port Lincoln from mixed biofouling samples. Mean and range is given as copy number/mg wet weight.

Time (month)	Depth (m)	Substrate	Pontoon	Prevalence	Mean	Range
1	1	Plate	A	17%	5,174	-
1	4	Plate	A	17%	3,340	-
1	1	Plate	B	17%	2,287	-
1	4	Plate	B	17%	650	-
1	1	Net	A	100%	2,239	1,042 – 3,494
1	4	Net	A	67%	2,256	1,243 – 3,307
1	1	Net	B	67%	2,829	1,942 – 4,394
1	4	Net	B	100%	2,382	2,030 – 2,812
3	1	Plate	A	100%	731	392 – 1,622
3	4	Plate	A	100%	667	367 – 1,239
3	1	Plate	B	100%	950	337 – 2,322
3	4	Plate	B	100%	1,705	467 – 5,042
3	1	Net	A	100%	1,482	373 – 3,256
3	4	Net	A	100%	1,614	1,274 – 2,122
3	1	Net	B	100%	1,218	483 – 1,981
3	4	Net	B	100%	1,636	898 – 2,054

Table 5.5. Prevalence (%) and copy numbers of *M. avidus* at Port Lincoln from taxonomic morpho-groupings. Range and mean is given as copies/mg (wet weight).

Sample	n	%	Range	Mean
Bivalvia	57	68%	522 – 2,565	1,586
Amphipoda	14	93%	1,275 – 1,844	1,340
Bryozoa	6	100%	1,560 – 1,841	1,697
Polychaeta	5	100%	1,243 – 1,362	1,304
Ascidiacea	3	100%	1,290 – 1,316	1,304
Anthozoa	1	100%	-	1,291
Gastropoda	1	100%	-	1,299
Tunicata	1	100%	-	1,340
Cirripedia	1	100%	-	1,321

5.5 Discussion

Both *C. forsteri* and *C. orientalis* were detected in mixed biofouling samples and biofouling organisms from a lease site with documented blood fluke infections. Gill and gill mucus samples of SBT from the same lease were positive for *C. forsteri* and *C. orientalis* (Balli Garza et al. unpublished). Neither *Cardicola spp.* were found at the same time in the same sample of biofouling. *Cardicola orientalis* was present mainly in mixed biofouling samples at 1 m and collected during three months. *C. forsteri* was found in a wider range of organisms at 4 m depth, and except one sample, all at 3 months.

Presence of the blood fluke DNA in biofouling may be due to the presence of free living stages or an infected intermediate host. Biofouling may harbor the

intermediate hosts, which can be infected with the parasites (Colquitt et al. 2001; Cribb et al. 2011; Palacios-Abella et al. 2015; Shirakashi et al. 2016; Ogawa et al. 2017). Studies have shown that the prevalence of intermediate hosts can vary depending on seasonality, depth and substrate (Fitridge et al. 2012; Shirakashi et al. 2016). None of the positive samples were from known intermediate hosts of *Cardicola* and all polychaete samples tested negative, thus the detection of *C. forsteri* and *C. orientalis* DNA is most likely due to the presence of free living stages of the parasite, physically associated with other biofouling organisms. Since 2015, *C. forsteri* is the species more prevalent in SBT (Neumann et al. 2018) but the presence of *C. orientalis* DNA was also detected in biofouling.

An increase in the presence of *M. avidus* was seen at three months. Crosbie and Munday (1999) noted that decreases in bacterial diversity and load due to a change in temperature would impact Scuticociliate populations by affecting their food supply. Thus, in the absence of bacteria SBT may be preferentially colonised by *M. avidus* during the colder months of the year. This could be an explanation for the increase in *M. avidus* detected around SBT leases at three months when water temperatures are lower. Average water temperature for May was 17℃ compared to 13℃ in July for 2016. This also corresponds to the occurrence of swimmer syndrome, which has been reported to be more common in the winter months (Munday et al. 1997).

Another potential explanation for the increase in prevalence of *M. avidus* around SBT pontoons during colder months could be difference in temperature between water and tuna. The eye and brain of tuna were 5-6℃ warmer than sea water temperature, for example in tuna from 20°C water, brain temperatures averaged about 27℃, eye temperatures about 26°C and maximum muscle temperatures about 30°C.

(Linthicum and Carey, 1972) The warmer temperatures in tuna may be a more ideal environment for *M. avidus* than colder sea water temperatures.

Most blood fluke positive samples were found at three months. This may either mean that the parasite DNA in biofouling increases with ranching time, possibly due to increasing infections of SBT or that the parasites associate with more mature biofouling. While the latter may be true for *M. avidus*, it is more likely that blood fluke DNA in biofouling was related to an increase in the presence of free life stages in the environment. This could be due to release of miracidia from infected SBT gills, usually related to the intensity of infection or an increase in the release of cercariae from the intermediate host. The factors affecting release of *C. forsteri* or *C. orientalis* cercariae from the intermediate host are not understood, however it is known that under laboratory conditions the polychaete *Terebella* spp. can release up to 1500 *C. opisthorchis* cercariae a day (Sugihara et al 2017).

While pontoon had no effect on the presence of *M. avidus* DNA in the biofouling, *C. forsteri* DNA was mostly detected in the biofouling from pontoon A. This is possibly due to higher levels of blood fluke infection in pontoon A as suggested by high SBT mortality in May in that pontoon. SBT infection model with *C. forsteri* had bimodal pattern when the infections were untreated, with the main infection occurring at day 14 and day 55 post-transfer (Aiken et al. 2009). While the pattern could have changed now due to on-going praziquantel treatment it is possible that the SBT mortality in May was caused by blood fluke infection. Whilst it would be beneficial to investigate the presence of parasites in fish from the same pontoons as sampled, it was not possible in our study. Future research is needed to investigate if there is a relationship between the presence of parasitic DNA in biofouling and health of SBT ranched in close proximity to biofouling.

Blood fluke DNA was more common in biofouling growing on the plates than on the organisms from the net. However, substrate had no effect on the detection of *M. avidus* DNA, suggesting Scuticociliates are common in the marine environment, including biofouling. The choice of substrate used for pathogen monitoring maybe pathogen specific. Similarly, depth (1 or 4 m) did not have an effect on the detection of *M. avidus* DNA. However almost all samples positive for blood fluke DNA were from 4 m depth, suggesting 4 m is a preferred depth for monitoring presence of SBT pathogens in biofouling.

Biofouling samples were separated into broad taxonomic groups to help identify reservoirs of infection for *M. avidus*. Bivalves, e.g. zebra mussels *Dreissena polymorpha* have been shown to harbor Scuticociliates inside their tissues (Molloy et al. 1996). Scuticociliates have also been associated with mortalities in an oyster hatchery (Plunket and Hidu 1978). However, for the purpose of this study, we could not conclusively determine presence or absence of *M. avidus* within bivalve organs in an environmental sample due to the possibility they were filter feeding (Bott et al., 2008). Prevalence of *M. avidus* was widespread in biofouling samples, with detection seen in all invertebrate taxa. This confirms that biofouling is a potential reservoir of this Scuticociliate. However, as *M. avidus* is a facultative parasite, it is unknown if there is a relationship between the presence of *M. avidus* DNA in the biofouling and swimmer syndrome in SBT.

The use of molecular methods to study biofouling assemblages is a relatively recent area of research, used in particular to identify invasive species and understand biodiversity (Pochon et al. 2015; Zaiko et al. 2016). Our approach was to monitor for the presence of *C. forsteri, C. orientalis* and *M. avidus* in DNA extracted from naturally occurring biofouling organisms and mixed biofouling collected from SBT pontoons.

Monitoring for environmental reservoirs of infection around aquaculture leases may lead to non-invasive monitoring of pathogens. This approach could also be used to monitor for specific pathogens affecting ranched fish. With continued advancements, approaches to environmental detection of aquaculture pathogens could become an effective and efficient tool to manage fish health and to develop future management strategies for the aquaculture industry.

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Chapter six

General Discussion

This research project focused on the detection of *Cardicola forsteri, C. orientalis* and *Miamiensis avidus* in Southern Bluefin Tuna (SBT) lethal and non-lethal samples, as well as in biofouling samples. Chapter 2 is a literature review of the diseases affecting SBT, Pacific Bluefin Tuna (PBT) and Northern Bluefin Tuna (NBT). In Chapter 3, conventional diagnostic methods used routinely for the diagnosis of *Cardicola* were compared to molecular diagnosis using real-time qPCR species-specific assays, allowing the detection of *Cardicola* and the differentiation between *C. forsteri* and *C. orientalis*, giving a quantitative result for most of the samples. In the same Chapter, results from hearts and gills using lethal sampling techniques, were compared against results from serum, gill biopsies and gill mucus swabs using non-lethal sampling methods. In Chapter 4, samples of cerebrospinal fluid from SBT presenting swimmer syndrome were analysed for the presence of Scuticociliates, using conventional observational techniques and end point PCR and sequencing. Based on the findings of Chapters 3 and 4 and using the best methods identified in these Chapters, Chapter 5 was developed. To improve the knowledge of the relationship between parasite, intermediate host and definitive host, biofouling samples obtained near the tuna pontoons were analysed using real-time qPCR with species-specific primers for *M. avidus*, *C. forsteri* and *C. orientalis* (Chapter 3) were used in this study.

The results obtained in this research project evaluate the potential that different diagnostic techniques have, showing the limitations of conventional diagnosis as well as, the advantages molecular techniques present. It also demonstrates that non-lethal sampling methods could be a viable option for the diagnosis of *Cardicola* in SBT, improving fish welfare by detecting the parasite in the fish before the onset of the diseases, as non-lethal sampling can be used for monitoring the state of health of the

farm, without the need to sacrifice fish, which also reduces economical losses due to sacrificing fish before harvesting season, in order to obtain samples for diagnosis.

Additionally, for the first time, this research project provided evidence of the presence of *M. avidus* in SBT exhibiting swimmer syndrome. Previous to this research, only *Uronema nigricans* had been associated with swimmer syndrome in SBT (Munday et al., 1997).

Finally, these findings provide deeper insights into the reservoir – pathogen – host interactions in ranched SBT, providing basic information about possible sources of infection with *M. avidus* and blood fluke from the genus *Cardicola*: *C. forsteri* and *C. orientalis*. As results showed that the parasites can be detected in the environment, these findings can contribute to the development of preventive practices, reducing the presence of these pathogens near the tuna pontoons and possibly monitoring the presence of some parasites on lease sites using biofouling samples.

6.1 Sampling techniques and diagnostic methods in aquaculture

Diagnostic techniques usually involve lethal sampling and laboratory analysis, which affects the logistics and the design of fish health surveillance programs. In aquaculture, surveillance and disease control are the key factors to stop further spread of pathogens, avoiding possible outbreaks and allowing fast decisions and rapid actions when needed to contain infective microorganisms (Adams, 1999; Adams and Thompson, 2011). Currently, to perform epidemiological surveillance, tuna industry requires lethal sampling which results in economic losses, as the sampled fish cannot always be sold due to a withdrawal period for praziquantel. This withholding period is established by the prescribing veterinarian, who indicates to the company, the time frame when SBT cannot be slaughtered for consumption purposes.

Monitoring the state of health of the ranched fish includes screening diseased fish and fish that are apparently healthy, allowing the detection of early stages of infection (Adams and Thompson, 2011). Because the SBT stock is limited and there is a quota of how many fish can be caught each year, there is a restricted number of ranched SBT that can be sampled, making routine monitoring of the state of SBT health impractical. In this project, lethal and non-lethal sampling methods used in the diagnosis of *C. forsteri* and *C. orientalis* gave similar results. However, for these species lethal sampling along with conventional diagnosis is limited to the detection of only two different life stages (adults and eggs) in hearts and gills, relaying on the microscopic observation of adults present in heart flushes samples, and number of eggs in the gill filaments. Only these two organs are analysed for the detection of *Cardicola*, as analysing more would be too time consuming and impractical to perform in more than a couple of fish. Therefore, areas with high concentration of other life stages can be easily missed, not reflecting the real level of infestation in the fish.

Non-lethal sampling methods can be combined with molecular diagnostic techniques, allowing the detection of different life stages of the parasites. Molecular assays detect genetic material, parasites do not need to be physically intact as for morphological identification and they require smaller portions of tissue or less amount of sample and, in many cases, they can be more sensitivity, detecting low concentrations of the pathogen or more specific, allowing the differentiation between different species (Johnson et al., 2005; Vetter et al., 2006). In this study serum, gill biopsies and gill mucus were used as non-lethal samples and the diagnosis was performed using real time qPCR. Serum samples were the least reliable, as during the three years of sampling, only five samples were positive for *C. forsteri* and one for *C. orientalis*. Test of the blood collection method, obtaining serum and the laboratory

processing of the samples showed that neither serum nor laboratory processing interfered with DNA detection, therefore, the amount of *Cardicola* DNA in the blood was not representative of the presence of *Cardicola* adults and eggs in heart or gills. Further studies using fish untreated with anthelmintics should be performed, to determine if the treatment interfered with the recovery of DNA from serum, as the presence of the parasite in the fish diminishes when the drug is administered (Hardy-Smith et al., 2012; Ishimaru et al., 2013; Polinski et al., 2014a; Shirakashi et al., 2012a). Different stages of infection could also be interfering with the results obtained, but testing this in the field would be very difficult, as there is no control over the different variables including when the fish get infected. Nevertheless, this could be done in Japan, as many companies have closed the life cycle of PBT, making easier to monitor the state of health of the fish during the different life stages, including the hatchery, as well as, monitor and control the conditions of the pontoons where the fish are kept.

Even though positive results were obtained using gill mucus, further studies are needed to increase the sensitivity of this method, for example to establish the area of the gills where mucus should be collected or the size of the sampling area and number of swabs taken from each fish. In this project, three samples were taken per fish, resulting most of the time in only one or two positive samples. Because the corresponding gill filaments were positive for eggs and for DNA of *Cardicola* using real time qPCR, it is possible that negative results from mucus were in fact false negatives (samples that tested negative when the fish was positive), which could be a consequence of the sampling method, or that the presence of *Cardicola* in gill mucus depends on other factors such as life stage of the parasite, for example detection of *Cardicola* DNA when cercariae are entering the fish thought the gills or miracidia are hatching from the eggs and emerging though the gills but not when eggs are present

in the gills. In addition to the diagnosis, gill swabbing could also help establish the route of entry or exit of *C. forsteri* and *C. orientalis*, as it has been determined with other fish pathogens such as Viral hemorrhagic septicemia virus in rainbow trout (Cornwell et al., 2013) and koi herpesvirus in carp (Monaghan et al., 2015).

Traditionally, the identification of pathogenic microorganisms has relied on the use of laboratory techniques, heavily depending on the detection, directly (such as bacterial isolation) or indirectly (detection of antibodies against the pathogen), of the infectious agent, nevertheless, the presence of such pathogens in the host might only be identified for a limited period of time, especially during latency periods when microorganisms can remain undetected by conventional diagnostic tools like indirect detection assays, making it easy to get false negatives (Brugere et al., 2017). Different diagnostic tests can be adapted and applied in surveillance activities and the assays are usually used to confirm the presence or absence of a disease (OIE, 2014a). However, epidemiological surveys can become challenging as assays need to be sensitive enough to detect low levels of infection, reducing false negatives, and they also have to present high specificity, reducing false positive results, finally, results have to be delivered in a quick and accurate way, determining the current state of health of the farm (Brugere et al., 2017).

The diagnosis of main diseases affecting ranched SBT is performed using lethal sampling along with conventional diagnostic methods, usually showing a limited sensitivity (Deveney et al., 2005; Munday et al., 2003). Therefore, it is necessary to develop highly sensitive and specific diagnostic tests, capable of detecting the presence of low loads of pathogens avoiding cross reaction with harmless microorganisms (Adams and Thompson, 2011; Brugere et al., 2017). Molecular diagnosis is more cost effective compared to conventional diagnostic techniques, in

many cases, it has shown higher sensitivity than conventional methods, taking less time to deliver results (Adams and Thompson, 2011; Hiney and Smith, 1998) and having the advantage of being able to differentiate between species and providing a quantification of the pathogen load.

In Chapter 3 of this research project, quantification of *C. forsteri* and *C. orientalis* was performed in all gill filaments, gill biopsies and serum, allowing a comparison between number of adult flukes, egg counts and DNA copy number per sample in individual fish. Results showed that there is a low correlation between number of adults in heart flushes, eggs in gill filaments and DNA copy number from the gills, similar to the results obtained from gill mucus. There are many factors that could influence the difference between results. One reason for this could be that the DNA quantification includes DNA from eggs and DNA of free life stages (miracidia or cercariae) present in the gills, adding to the DNA copy number present in gill filaments, making it inconsistent when compared with the egg number. Another reason could be the life stage of the eggs, increasing the DNA copy number over time, as the egg develops. Gill biopsies results showed that the top of the gill filament had higher concentrations of DNA of *C. forsteri*. These results indicate that gill biopsies as a non-lethal sampling methods and real time qPCR could be used for diagnosis of *Cardicola* as an alternative to conventional techniques.

Most of the serum samples analysed for this project were negative or had very low DNA copy number. In 2013, Polinski et al. detected a higher prevalence of *C. forsteri* and *C. orientalis* in serum of SBT, however, after this study, praziquantel treatment was incorporated as a routine practice in SBT industry in Australia. The reduction of adult flukes in the hearts of SBT as a consequence of the use of anthelmintic, could have an impact in the amount of free DNA circulating in the blood,

or in the quantity of other life stages that could be present in the blood, decreasing the DNA copy number obtained in serum.

In recent years, the OIE has identified molecular techniques as important diagnostic tools, making them as relevant as conventional techniques (De Andrade, 2011; OIE, 2003). Even though kits and protocols available for the detection of pathogens in fish using molecular diagnostic methods are still limited, the development of these tests is growing (De Andrade, 2011; OIE, 2003). Once a molecular assay has been validated, proving to be reliable and available, it is added to the "Aquatic Manual" of the OIE, which is constantly updated including new assays (OIE, 2003). Several tests using PCR for diagnosis of fish pathogens have already been tested with positive outcomes (Table 6.1), but standardization and validation of most of these methods still has to be done (Hiney and Smith, 1998; OIE, 2014a, 2014b, 2003).

Through validation processes, competence of diagnostic tests is evaluated. Validated tests must be capable of identifying positive and negative individuals, avoiding misdiagnosis by providing a false result, within the limits of the validated test (OIE, 2003). According to the Aquatic Manual of the OIE, in order to validate a molecular diagnostic test, sample collection, transportation and DNA extraction methods are crucial and they should be optimized depending on the sample type as well as target. In this project an attempt to optimize blood processing to serum as well as DNA extraction from serum were performed (Chapter 3), using spiked samples with known DNA concentration of *C. forsteri*. The blood collection, and processing of serum did not interfere with the DNA recovery. In other experiments, using again a known concentrations of *C. forsteri* DNA for spiking collected serum, proved that DNA extraction methods are suitable for detecting the presence of *C. forsteri* DNA in SBT serum. It is possible that other factors such as, praziquantel treatment, do have an

impact in the quantity of free DNA or other life stages that could be present in the blood, especially since praziquantel has been found to be and effective treatment against *Cardicola* in Bluefin Tuna.

Molecular methods can also be employed in the detection of pathogenic microorganisms in the environmental samples, such as biofouling (Pochon et al., 2015; Shirakashi et al., 2016; Tan et al., 2002). In order to infect SBT, both *M. avidus* and *Cardicola* have to be present near the tuna pontoons. In the case of Scuticociliates, this can be in the sediment under the tuna pontoons (Munday et al., 2003) or in biofilm structures (Shimeta et al., 2012), while intermediate hosts of fish blood flukes are commonly found in biofouling (Fitridge et al., 2012; Shirakashi et al., 2016). In this research project we identified the presence of *M. avidus, C. forsteri* and *C. orientalis* DNA in biofouling samples. Gill mucus samples collected from fish from this site were also positive for both species of *Cardicola.* Molecular analysis of biofouling samples showed all parasites had a temporal distribution, with *Cardicola* and *M. avidus* mainly found in three month-old samples. Swimmer syndrome has been reported from SBT lease sites during the coldest months, between May and November (Crosbie and Munday, 1997). Changes in bacterial populations due to water temperature differences such as water temperature dropping below 18°C, have been seen to modify Scuticociliates behaviour, switching from bacterivorous to histophagous (Crosbie and Munday, 1997; Munday et al., 1997).

Differences in spatial distribution was also noted, where *Cardicola* was present mainly in samples at four m depth, meanwhile, depth had no effect on *M. avidus* prevalence. A study has reported a difference between the presence of the intermediate host *Nicolea gracilibranchis* infected with *C. orientalis* recovered mostly between two and four m depth (Shirakashi et al., 2017), which is consistent with our

findings. In this Chapter, quantification of both *Cardicola* species as well as, *M. avidus* was also obtained from positive biofouling organisms, results did not show any correlation between DNA copy number, *Cardicola* species, depth and age of the biofouling (one or three month). Further biofouling studies need to be performed assessing differences between months during the year, water temperatures, different depths as well as, different sampling sites within the tuna pontoons, they will also need to establish if there is a relationship between the DNA copy number obtained, the age and depth of the biofouling, and the biofouling organism. Future studies will help to determine if the DNA copy number obtained from biofouling samples are correlated with outbreaks in SBT, these results will allow a better management of preventive programs.

These studies will also need to determine if the differences observed in older biofouling samples is a result of, the biofouling being more developed, being exposed for a longer period of time with SBT, or because of seasonal environmental factors. This will help determine how environmental changes such as water temperature influence the presence of these parasites near the tuna pontoons, it will also help identify if other biofouling substrates, for example ropes, floats and pontoon collars, have an impact in the development of an ecosystem that could promote the presence of *Cardicola* and *Miamiensis* and at the same time.

Further research could also identify possible intermediate hosts of *C. orientalis* in Australia, by detecting organisms positive for the presence of *C. orientalis*, allowing a further targeted study towards these organisms. Finally, it will help gather a better understanding of the behaviour and development of pathogens in the environment, contributing to the improvement of preventive actions adapting management strategies that could help limit future outbreaks.

Table 6.1. Examples of PCR- based tests used in the detection of fish pathogens from clinical samples.

Pathogen	Clinical sample	Fish species	Reference
Infectious pancreatic necrosis virus	Kidney	Atlantic halibut *Hippoglossus hippoglossus* (L)	Gahlawat et al., 2004
Koi herpesvirus	Gill swabs, faecal samples, skin mucus, anal and fin swabbing	Common carp *Cyprinus carpio*	Bergmann and Kempter, 2011; Monaghan et al., 2015
Nervous necrosis virus	Blood, kidney biopsy, spleen, liver, heart	Atlantic halibut *Hippoglossus hippoglossus* (L)	Korsnes et al., 2009
Viral hemorrhagic septicaemia virus	Gill, left pectoral fin	Golden shiners *Notemigonus crysoleucas* and fathead minnows *Pimephales promelas*	Cornwell et al., 2013
White sturgeon iridovirus	Pectoral fins	White sturgeon *Acipenser transmontanus*	Drennan et al., 2007
Aeromonas salmonicida	Spleen, kidney, gills, intestine, skin	Atlantic salmon *Salmo salar*	Du et al., 2017
Edwardsiella piscicida, E. tardia, E. piscicida-like sp.	Kidney	Catfish *Ictalurus punctatus*	Reichley et al., 2015
E. tardia	Kidney, liver, intestine, spleen, blood, skin mucus	Turbot *Scophthalmus maximus*	Castro et al., 2010
Flavobacterium psychrophilum	Spleen, kidney, eggs, ovarian fluid	Rainbow trout *Oncorhynchus mykiss* and Coho salmon *O. kisutch*	Baliarda et al., 2002
Photobacterium damselae	Spleen	Gilthead seabream *Sparus aurata*	Carraro et al., 2018
Renibacterium salmoninarum	Fin clips, gill snips, skin mucus, blood, kidney biopsy,	Chinook salmon Oncorhynchus tshawytscha	Elliott et al., 2015
Streptococcus agalactiae	Kidney aspiration, blood, nasal wash, gill mucus, faecal samples	Nile tilapia *Oreochromis niloticus* (L)	Tavares et al., 2016
Tenacibaculum maritimum	Skin mucus,	Turbot, Sole, Gilthead *Scophthalmus maximu, Solea senegalensis, Sparus auratus*	Núñez et al., 2004
Yersinia ruckeri	Blood	Rainbow trout *Oncorhynchus mykiss*	Altinok et al., 2001
Cichlidogyrus spp.	Gill mucus	Nile tilapia *Oreochromis niloticus* (L)	Ek-Huchim et al., 2012

6.2 Rapid diagnostic tests

It is important to be able to establish the presence of specific pathogens in a rapid way, being able to determine if the disease is present or absent, especially on leases where outbreaks can cause severe loses, therefore, a timely detection of microorganisms is of great importance. The development of point-of-care (POC) tests is of great interest in aquaculture and industry to obtain the results in real time and reduce the costs of surveillance program (Brugere et al., 2017; Stoot et al., 2014). The diagnosis of *Cardicola* and Scuticociliates causing swimmer syndrome in SBT is not an exception. The diagnosis of those conditions is based on direct observation of Scuticociliates or adult flukes or eggs in the case of *Cardicola*, using microscopy techniques (Aiken et al., 2006; Munday et al., 1997) which could be impractical in the field, being time consuming and requiring experience. Contrarily, POC tests deliver results in a short period of time, being easy to use and without the need of complex equipment.

POC tests are *in vitro* diagnostic assays that can be carried out at or close to the site where the affected individuals are (International Organization for Standardization, 2016). Not limited to a particular location and not requiring specific facilities where they need to be performed, POC tests have the flexibility to be used on-site, giving immediate results and allowing a fast implementation of procedures for the control of diseases, avoiding economical losses (International Organization for Standardization, 2016; Kozel and Burnham-Marusich, 2017). With a growing need to design and create tests that adjust to specific needs, that will allow the detection of different pathogens without sacrificing sensibility of specificity, more POC molecular methods for commercial purposes will be develop during coming years (St John and Price, 2014).

POC tests are convenient, especially in a fast pace working environments, where available specialized staff is limited or where decisions have to be made quickly (Austin and Newaj-Fyzul, 2017; Kozel and Burnham-Marusich, 2017). These tests have been available for approximately 40 years and were initially used for biochemistry and hematology. POC tests continue to become more popular than conventional laboratory testing, as they are less expensive and more sensitive and precise, while requiring less equipment (St John and Price, 2014). With the capacity to adjust the analysis protocols, they present the advantage to be used directly in the field, rapid sample processing, minimum possible loss caused by transportation, sample degradation or mishandling (Stoot et al., 2014).

Rapid tests can be divided into two main categories, the first use portable devices which can provide quantitative and qualitative data (St John and Price, 2014), such as the ones used for blood physiology (Stoot et al., 2014); The second ones are basically scale down bench-top laboratory equipment, making them less complex to use. Devices using molecular techniques like PCR fall into this second category (St John and Price, 2014). Other methods such as latex agglutination tests can be found commercially (Romalde et al., 1995), but they have the limitation of only being capable of detecting specific microorganisms (Brugere et al., 2017).

POC tests used for the diagnosis of microorganisms, are already in use in aquaculture, many of them are based on molecular techniques (Austin and Newaj-Fyzul, 2017; Bergmann and Kempter, 2011; Núñez et al., 2004; Soliman and El-Matbouli, 2005). Many traditional methods are complicated and time consuming, delaying the diagnosis and treatment, therefore, it is important to have a more effective approach (Soliman and El-Matbouli, 2005) (Table 6.2). Most of the POC tests used in aquaculture have been employed to evaluate the parameters of teleost fish blood

physiology, but most of the tests lack of validation, therefore, it is important to design specific tools that can be used for this purposes (Stoot et al., 2014).

Nucleic acid- based tests, such as PCR and real time qPCR have proven to be an important tool, widely used in different life science areas (Notomi et al., 2000), they have also shown to be more sensitive than conventional diagnostic techniques, being flexible enough to be adapted for the detection of multiple pathogens (Brugere et al., 2017; St John and Price, 2014). Based on molecular techniques, Loop Mediated Isothermal Amplification (LAMP) utilizes a similar technology to PCR, where different sets of primers hybridize with six specific regions of the target DNA. DNA synthesis is performed using *Bst* DNA polymerase large fragment, DNA replication takes place under isothermal conditions ranging between 60-65^{O}C, producing large amounts of DNA with an increased stem in less than 60 minutes, positive samples are detected by turbidity or with a dye like SYBR Green (Notomi et al., 2000). This assay is highly specific, faster and more sensitive that end point PCR, with the advantage of being suited for field conditions, as it does not require machines like thermocyclers (Adams and Thompson, 2011).

Table 6.2. Differences between diagnostic methods in aquaculture.

Diagnostic method	Type of sample	Quantitative detection	Risk of contamination	Laboratory special requirements	Operation difficulty	Multiple samples in single reaction	Field applications
Culture	Serum Tissue Culture	Easy	Low	Yes	Easy	Possible	Difficult
ELISA	Serum	Easy	Low	Yes	Average	Difficult	Difficult
Histology	Tissue	Difficult	Low	Yes	Easy	Possible	Difficult
Indirect and direct florescent antibody	Serum Tissue Culture	Easy	Low	Yes	Average	Difficult	Difficult
Immunohistochemistry	Tissue	Difficult	Low	Yes	Average to difficult	Difficult	Difficult
Loop-mediated amplification	Tissue Serum Mucus	Possible	High	No	Easy	Difficult	Possible
Microscopy	Smears	Difficult	Low	No	Easy	Possible	Possible
PCR	Tissue Serum Mucus	Difficult	High	Yes	Average	Possible	Possible
Real time PCR	Tissue Serum Mucus	Possible	High	Yes	Difficult	Possible	Possible
Western blot and dot blot	Serum	Difficult	Low	Yes	Average to difficult	Possible	Difficult

LAMP on-site tests have been used for the detection of different pathogens in aquaculture such as bacteria and virus, providing a precise, rapid, and cost-effective field diagnostic technique (Marlowe Caipang et al., 2015) (Table 6.3), it has successfully been used for the detection of at least one fish parasite, using water and gill tissue samples (Picón-Camacho et al., 2013). Being a highly sensitive and specific assay, as it uses the same resources as PCR, it can easily be adapted for the detection of parasites such as *C. forsteri*, *C. orientalis* and *M. avidus* in both lethal and non-lethal SBT samples, or as a surveillance tool identifying the presence of pathogens affecting SBT in environmental samples, such as water or biofouling samples. Further studies will need to be carried on directly in the field to evaluate the performance of these assays, as they could also be implemented as screening tests identifying *Cardicola* positive biofouling organisms and along with other diagnostic methods determine possible intermediate hosts of *C. orientalis* in Australia.

Table 6.3. Examples of pathogens detected with Loop mediated isothermal amplification (LAMP) assays.

Pathogen	Species	Reference
Cyprinid herpesvirus 2	Crucian Carp *Carassius carassius*	He et al., 2013
Infectious hematopoietic necrosis virus	Rainbow Trout *Oncorhynchus mykiss*	Gunimaladevi et al., 2005
Infectious spleen and kidney necrosis virus	Nile Tilapia *Oerochromis niloticus and* Red Hybrid Tilapia, *O. niloticus* x *O. mossambicus*	Suebsing et al., 2016
Koi herpesvirus	Common Carp *Cyprinus carpio* and Koi Fish *C. rubrofuscus*	Soliman and El-Matbouli, 2005
White spot syndrome virus	Asian Tiger Shrimp *Penaeus monodon*	Jaroenram et al., 2009
Edwardsiella ictaluri	Catfish *Ictalurus punctatus*	Yeh et al., 2005
Edwadrsella tarda	Japanese Flounder *Paralichthys olivaceus*	Savan et al., 2004
Vibrio vulnificus	Bacterial culture	Ren et al., 2009
Amyloodinium ocellatum	Parasite culture	Picón-Camacho et al., 2013

In the course of this study, several sampling methods were evaluated along with conventional and molecular diagnosis. Conventional techniques are routinely used by industry; therefore, it was necessary to evaluate the sensitivity and specificity of non-lethal sampling and molecular diagnosis for the detection of *Cardicola*, assuring diagnostic results with positive relative percentages similar or higher when compared to conventional methods. Serum samples which are commonly used in a wide variety of fish species as non-lethal samples, have been used in past experiments, providing promising results, identifying the presence of *C. forsteri* and *C. orientalis* in SBT serum (Polinski et al., 2013), but serum samples processed for this research project showed poor sensitivity. Obtaining low DNA copy number of *Cardicola* in serum in this study, could be a result of the current implementation of preventive measurements, such as praziquantel treatment. Future studies will need to establish the relationship between

the use of praziquantel, adults present in the heart and DNA copy number from serum, as they will help determine how treatment impacts the life cycle of *Cardicola* in SBT.

As a result of serum poor performance in diagnosis, mucus samples were tested as an alternative non-lethal method Preliminary evaluation showed promising results, but further assessment will need to be done in order to determine if this samples truthy reflect the level of infection of the fish or, if they detect free living stages that do not necessarily get to infect the tuna. Results also demonstrated that is will be important to collect more than one sample per fish, as *Cardicola* was not always detected in all collected samples. Other non-lethal sampling methods such as gill biopsies and gill filament analysis combined with molecular diagnostic techniques, showed encouraging results, which are comparable with the ones obtained using lethal sampling, presenting the advantage of being able to differentiate between *Cardicola* species in one single process, using real time qPCR.

To make these results applicable to other aquaculture industries, the sampling and diagnostic techniques used in this project will need to be evaluated for other fish species and other pathogens, the detection of other microorganisms using these techniques will depend on the organ which is affected, and on the possibility to take non-lethal samples for its diagnosis. The non-lethal and lethal sampling methods used in this project are commonly used in other species and they could be combined with screening techniques such as LAMP or field-based qPCR for surveillance of other pathogens in ranched fish as well as a monitoring tool for exotic diseases in farms, contributing in the development of disease control programs, limiting possible outbreaks.

In the course of this study, analysis of biofouling showed that it is possible to detect *Cardicola* and *M. avidus* in biofouling organisms. Even though these are

encouraging results, they must be taken with caution, future research will need to be performed to determine if the PCR positive samples are due to the presence of free living stages of *Cardicola* or if other terebellid species are capable of harbouring *Cardicola* as intermediate hosts. This research will also establish the conditions when *Cardicola* and *M. avidus* are most prominent, helping to determine if preventive actions are needed, as the assays used during this project can be adapted as part of surveillance programs.

In conclusion, this research project provided evidence of the efficiency of non-lethal samples and real time qPCR assays, used for the diagnosis of *C. forsteri* and *C. orientalis* in SBT, it also established the presence of *M. avidus* in fish exhibiting swimmer syndrome as possible causative agents. Additionally, preliminary results showed the possible use of real time qPCR techniques in the surveillance of *Cardicola* and *M. avidus* in SBT pontoons, using environmental samples such as biofouling, justifying further investigations of the relationship between *Cardicola* and biofouling organisms in Australia.

Appendix

Normal distribution and Non-parametric tests

Q-Q plot and histogram

Observations of chapter 3 were tested to determine if they had a normal distribution. The observations tested were adults present in the heart, eggs present per milligram of gill filament, *C. forsteri* DNA copy number per milligram of gill filament, *C. orientalis* DNA copy number per milligram of gill filament, serum positive for *C. forsteri* and serum positive for *C. orientalis*.

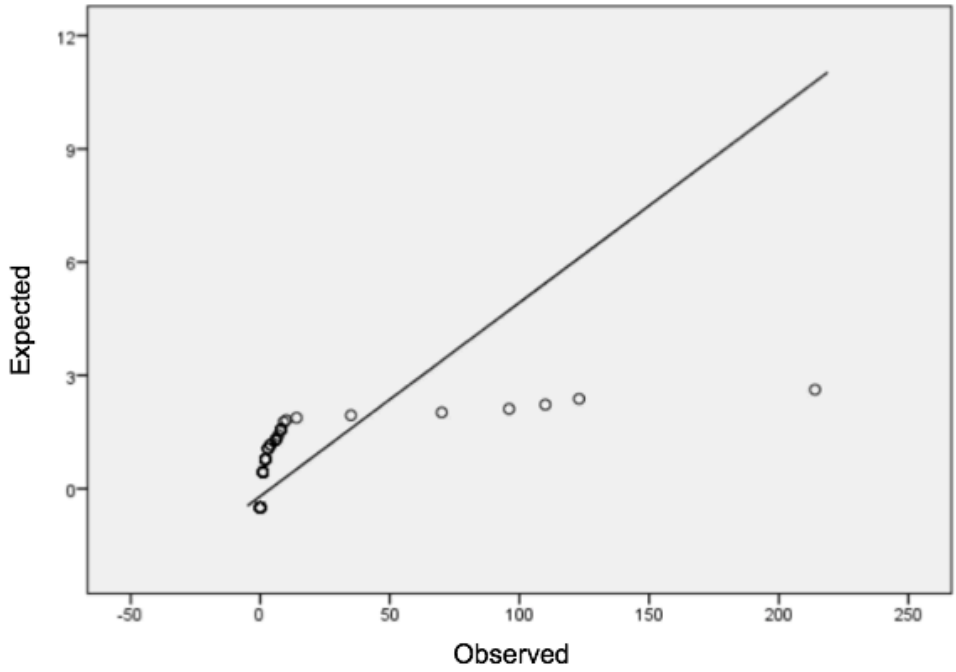

Figure 5.1 Q-Q plot for adults observed in heart against expected adults in heart.

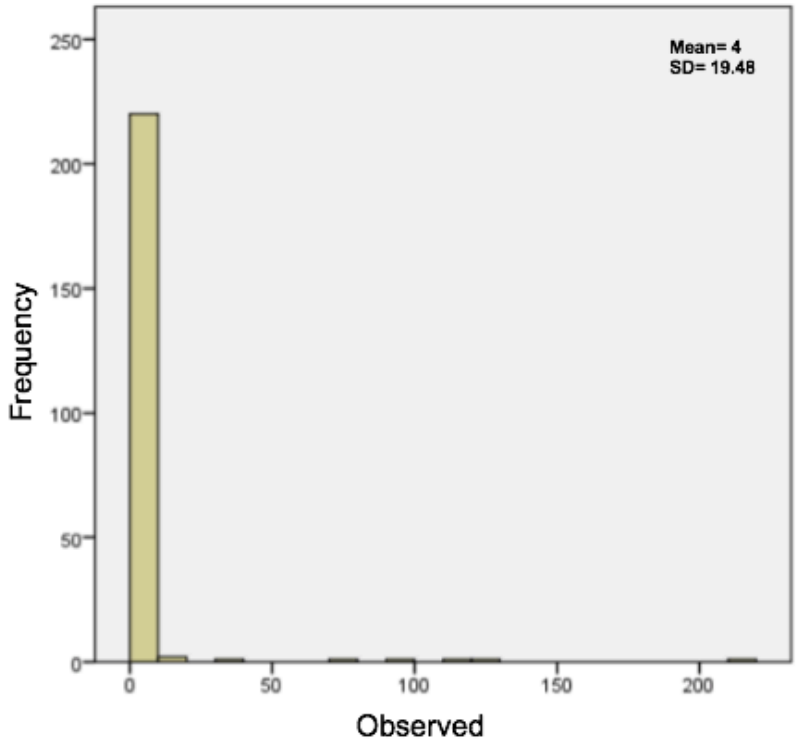

Figure 5.2 Histogram showing frequencies of adults observed in hearts.

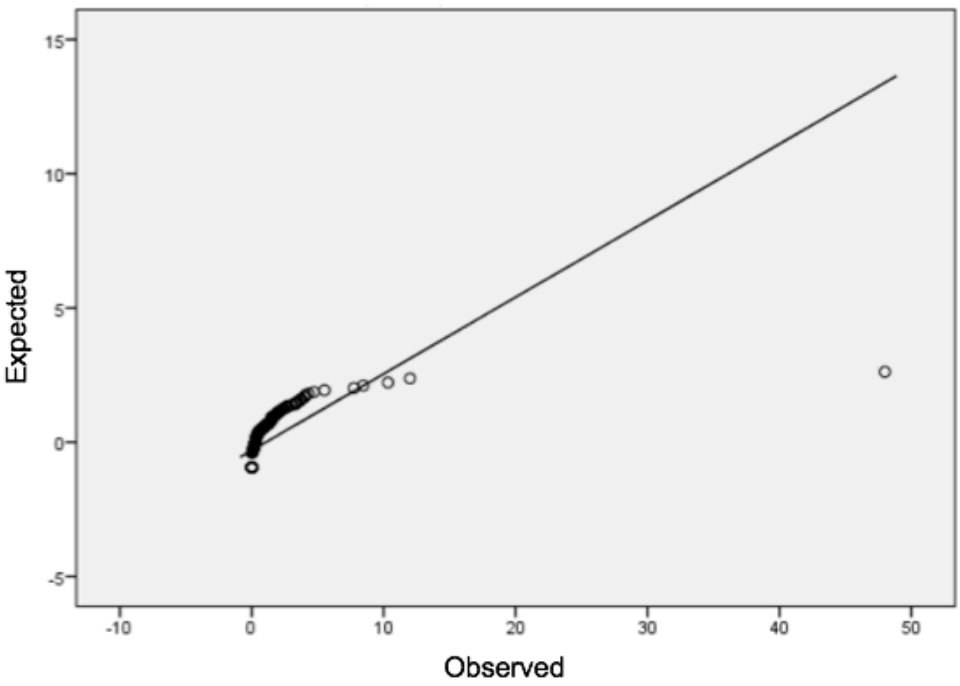

Figure 5.3 Q-Q plot of number of eggs observed per milligram of gill filament and expected value.

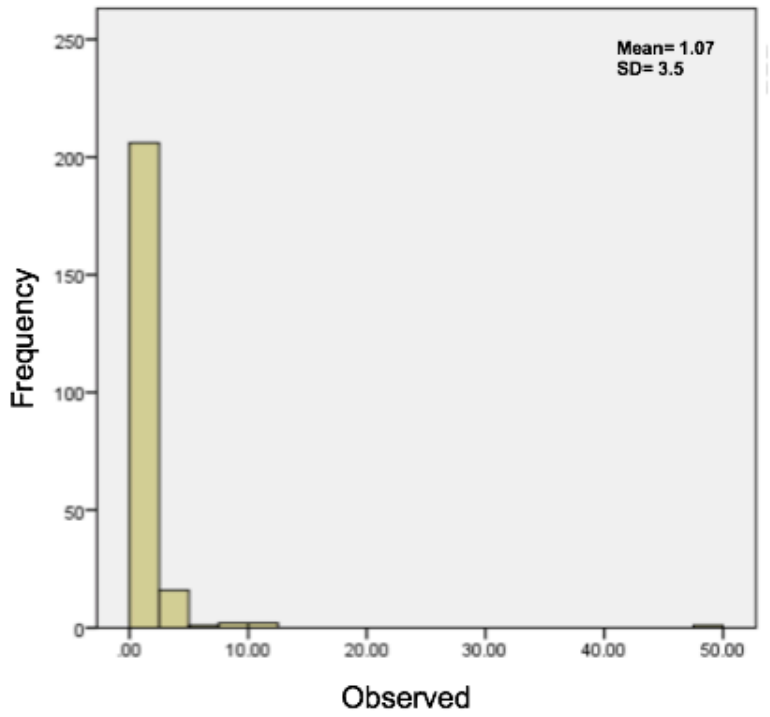

Figure 5.4 Histogram showing frequencies of eggs per milligram of gill filament.

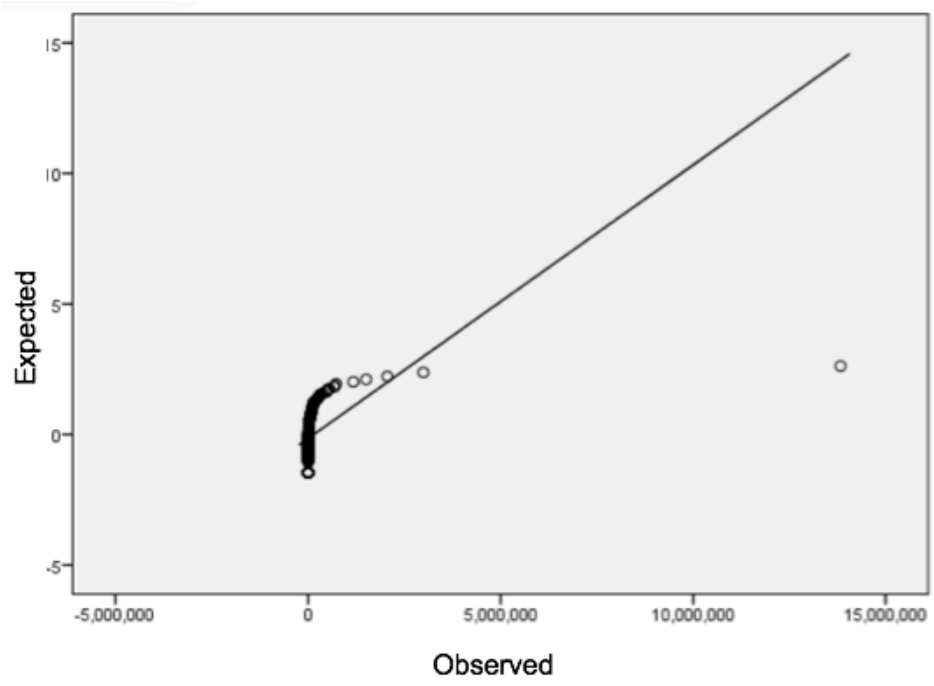

Figure 5.5 Q-Q plot of *C. forsteri* DNA copy number per milligram of gill filament observed and expected value.

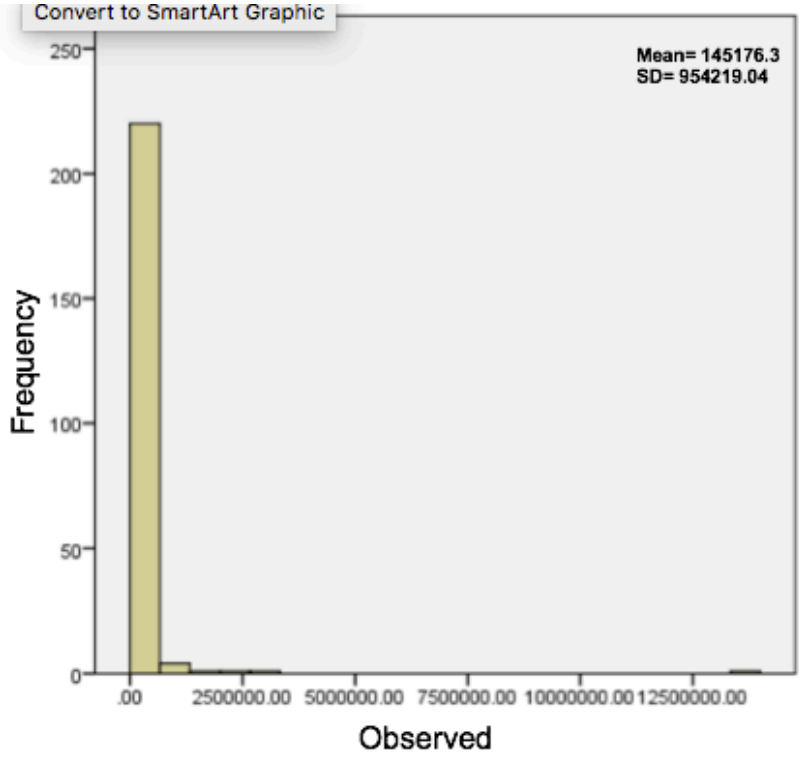

Figure 5.6 Histogram showing frequencies of *C. forsteri* DNA per milligram of gill filament.

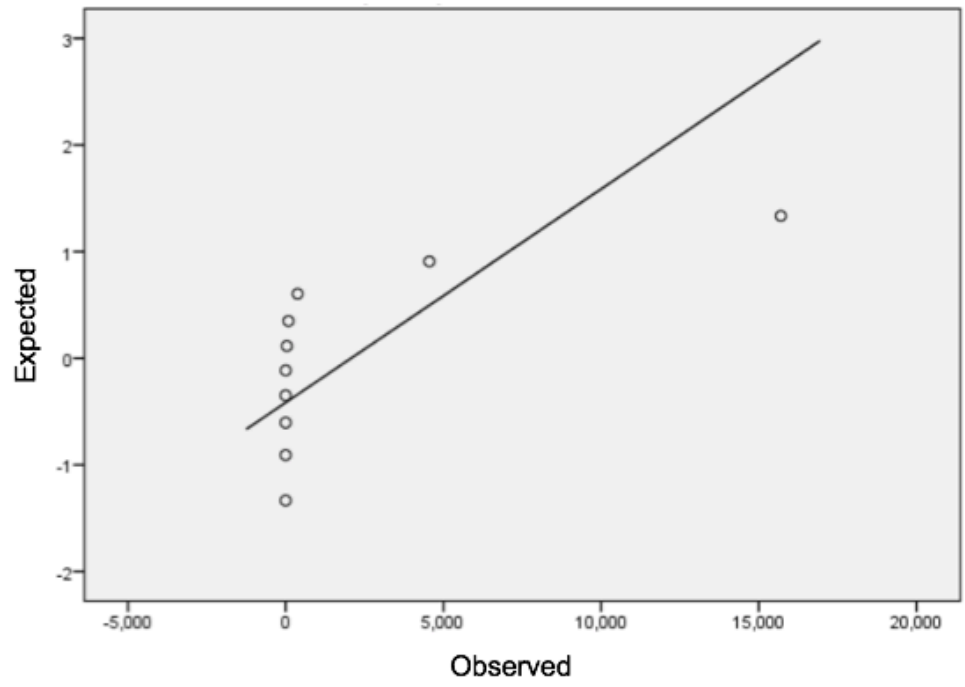

Figure 5.7 Q-Q plot of *C. orientalis* DNA copy number per milligram of gill filament observed and expected value.

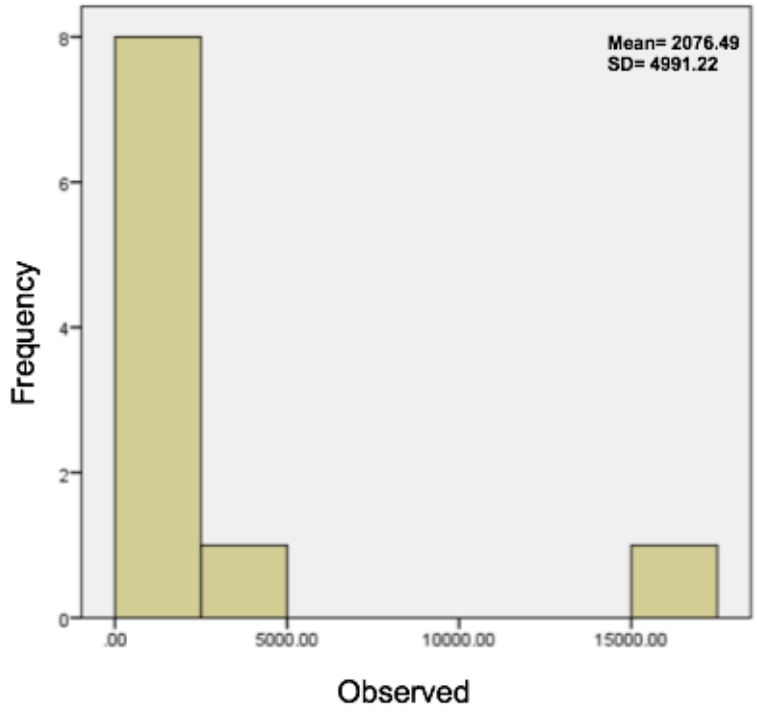

Figure 5.8 Histogram showing frequencies of *C. orientalis* DNA per milligram of gill filament.

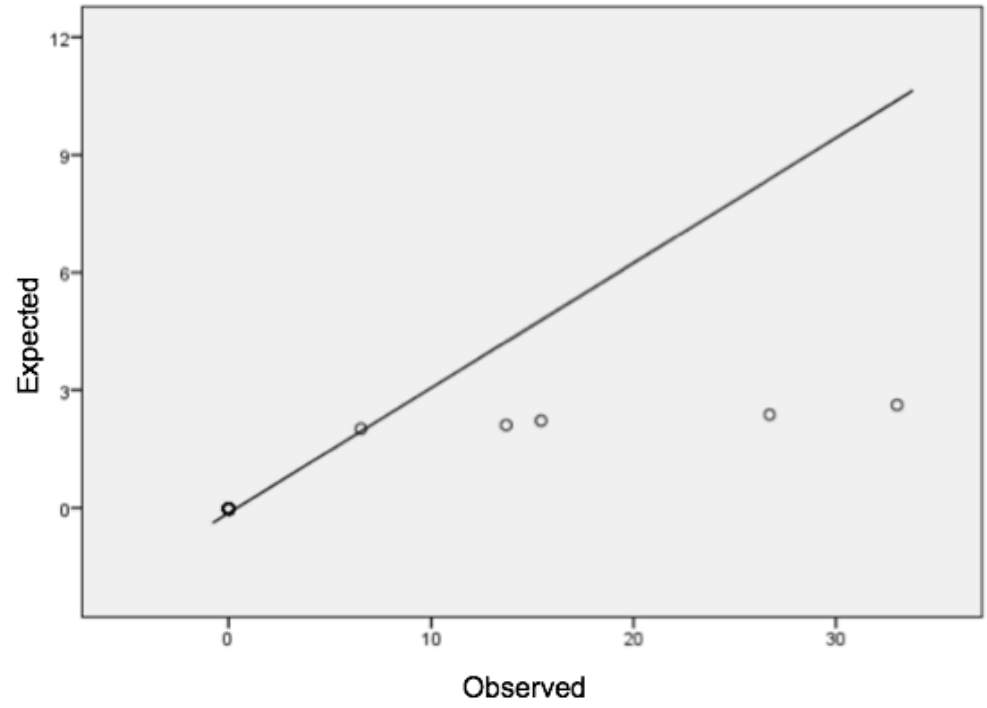

Figure 5.9 Q-Q plot of serum positive for *C. forsteri* observed and expected value.

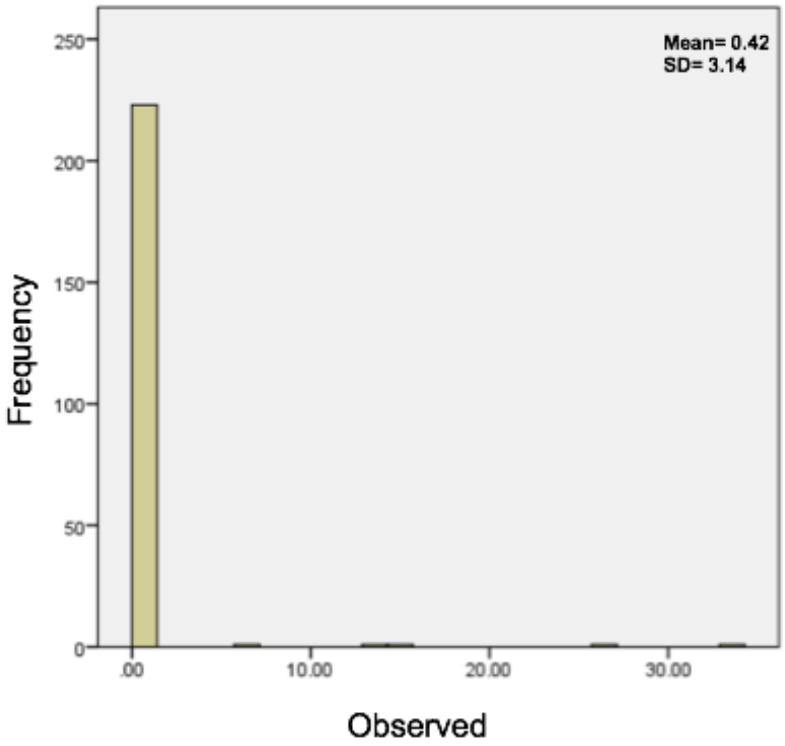

Figure 5.10 Histogram showing frequencies of serum positive for *C. forsteri*.

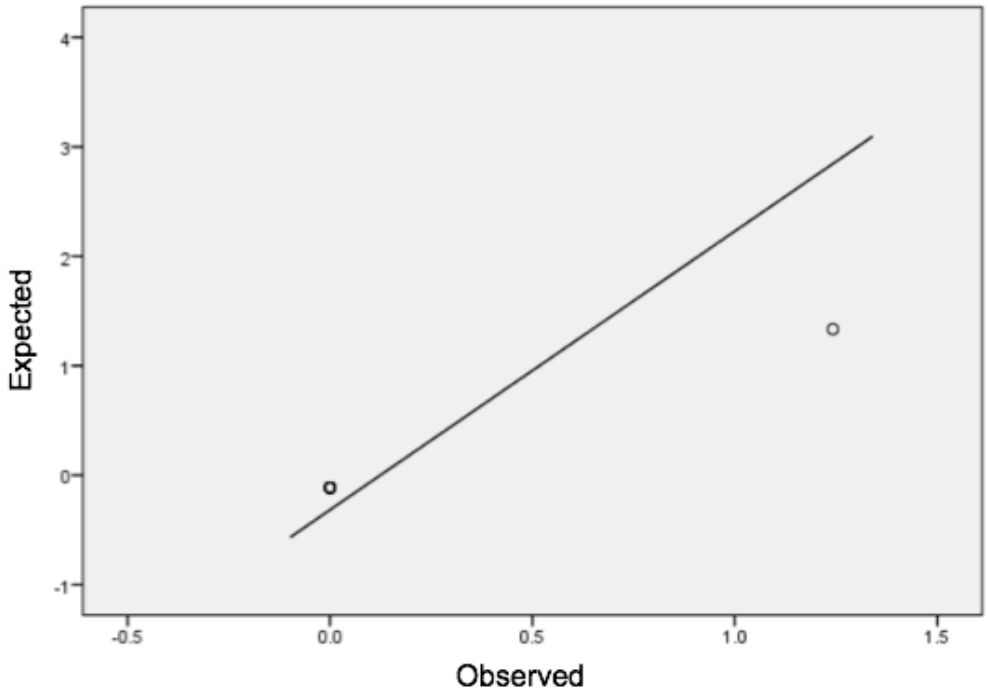

Figure 5.11 Q-Q plot of serum positive for *C. orientalis* observed and expected value.

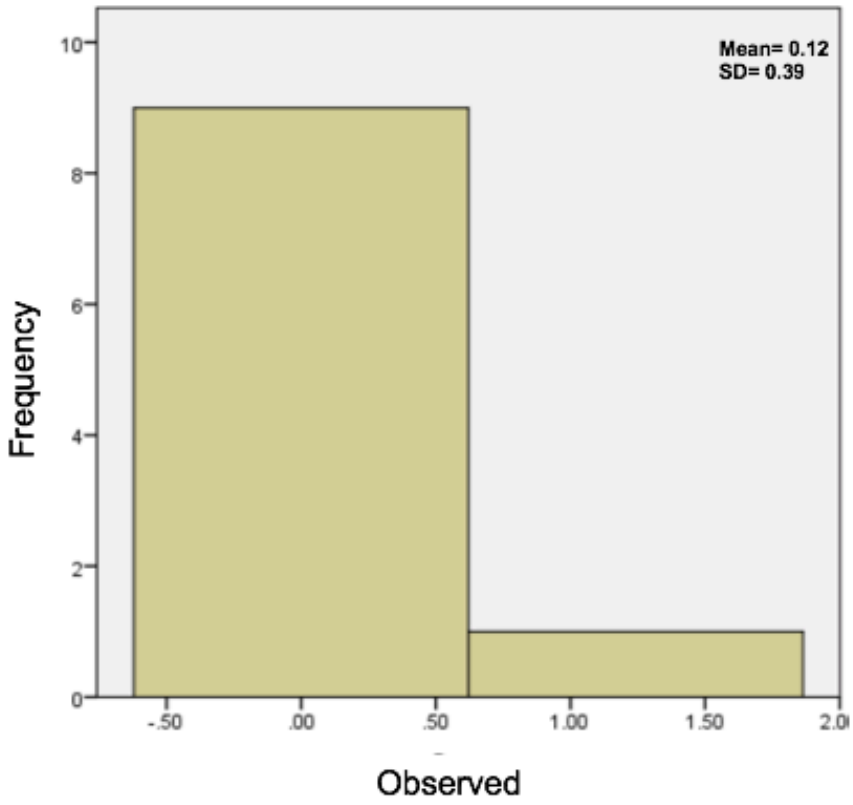

Figure 5.12 Histogram showing frequencies of serum positive for *C. orientalis*.

Wilcoxon signed-rank test for independent variables

As samples (observations) violate the assumptions for parametric tests, observations do not have a normal distribution, they present a right-skew, samples were transformed and Wilcoxon signed-rank test for independent variables was used to analyse and compare between the presence of *C. forsteri* and *C. orientalis* in different lethal and non-lethal samples, using SPSS software (IBM SPSS Software, NY, USA).

Median analyses showed no significant differences between both species during 2015, 2016 and 2017. Results of the tests showed no significant differences between lethal and non-lethal sampling method, except when comparing adults

present in the heart and DNA copy number per mL of serum, where both parametric and non-parametric tests show a significant positive correlation with a *p-value* < 0.05 using parametric and non-parametric tests.

Positive results for *C. forsteri* from 2015, 2016 and 2017 were further analysed, with the intention to find differences in the presence of this *Cardicola* specie. *C. orientalis* was not considered as it was found in fewer samples. Results of *C. forsteri* samples showed that *C. forsteri* has a normal distribution each year with no tendencies. The results obtained using non-parametric tests show similar results to the ones obtained using parametric tests.

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